



PCT

特許協力条約に基づいて公開された国際出願

<p>(51) 国際特許分類7 C12N 15/86, 15/45, 7/01 // 5/10</p>	<p>A1</p>	<p>(11) 国際公開番号 WO00/70070</p> <p>(43) 国際公開日 2000年11月23日(23.11.00)</p>
<p>(21) 国際出願番号 PCT/JP00/03195</p> <p>(22) 国際出願日 2000年5月18日(18.05.00)</p> <p>(30) 優先権データ 特願平11/200739 1999年5月18日(18.05.99) JP</p> <p>(71) 出願人 (米国を除くすべての指定国について) 株式会社 ディナベック研究所 (DनावEC RESEARCH INC.)(JP/JP) 〒305-0856 茨城県つくば市観音台1丁目25番11号 Ibaraki, (JP)</p> <p>(72) 発明者 ; および</p> <p>(75) 発明者 / 出願人 (米国についてのみ) 李 海鷗(LI, Hai-Ou)(CN/JP) 朱 亜峰(SHU, Tsugumine)(JP/JP) 上田泰次(UEDA, Yasuji)(JP/JP) 長谷川護(HASEGAWA, Mamoru)(JP/JP) 飯田章博(IIDA, Akihiro)(JP/JP) 時任文乃(TOKITOU, Fumio)(JP/JP) 平田隆洋(HIRATA, Takahiro)(JP/JP) 徳炭 剛(TOKUSUMI, Tsuyoshi)(JP/JP) 〒305-0856 茨城県つくば市観音台1丁目25番11号 株式会社 ディナベック研究所内 Ibaraki, (JP) 隈 秀和(KUMA, Hidekazu)(JP/JP) 〒301-0044 茨城県竜ヶ崎市の小柴4-6-3 久光製薬株式会社 竜ヶ崎ハイイツA203 Ibaraki, (JP)</p>	<p>浅川 誠(ASAKAWA, Makoto)(JP/JP) 〒561-0825 大阪府豊中市二葉町3-2-1 シオノギ神崎川寮319号室 Osaka, (JP)</p> <p>(74) 代理人 清水初志, 外(SHIMIZU, Hatsushi et al.) 〒300-0847 茨城県土浦市御町1-1-1 関鉄つくばビル6階 Ibaraki, (JP)</p> <p>(81) 指定国 AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, 欧州特許 (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI特許 (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG), ARIPO特許 (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), ユーラシア特許 (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM)</p> <p>添付公開書類 国際調査報告書 請求の範囲の補正の期限前の公開; 補正書受領の際には再公開される。</p>	
<p>(54)Title: PARAMYXOVIRIDAE VIRUS VECTOR DEFECTIVE IN ENVELOPE GENE</p> <p>(54)発明の名称 エンペローブ遺伝子欠損パラミクソ科ウイルスベクター</p> <p>(57) Abstract Virus virions defective in F gene are successfully collected by using a Sendai virus genomic cDNA with deletion of F gene. Further, infectious viral particles defective in F gene are successfully constructed by using F-expression cells as helper cells. Also, virus virions defective in F gene and HN gene are successfully collected by using a virus genomic cDNA with deletion of both of F gene and HN gene. Further, infectious viral particles defective in F gene and HN gene are successfully produced by using F- and HN-expression cells as helper cells. A virus being defective in F gene and HN gene and having F protein is constructed by using F-expression cells as helper cells. Further, a VSV-G pseudo type virus is successfully constructed by using VSV-G-expression cells. Techniques for constructing these defective viruses contribute to the development of vectors of <i>Paramyxoviridae</i> usable in gene therapy.</p>		

BEST AVAILABLE COPY

(57)要約

F 遺伝子を欠失させたセンダイウイルスゲノムcDNAを用いて、F 遺伝子を欠損したウイルスビリオンを回収することに成功した。さらに、F 発現細胞をヘルパー細胞として用いて、F 遺伝子欠損型の感染性ウイルス粒子を作製することに成功した。また、F およびHN 遺伝子の両方を欠失させたウイルスゲノムcDNAを用いて、F およびHN 遺伝子を欠損したウイルスビリオンを回収することに成功した。さらに、F およびHN 発現細胞をヘルパー細胞として用いて、F、HN 遺伝子欠損型の感染性ウイルス粒子を作製することに成功した。また、F 発現細胞をヘルパー細胞として用いて、F 蛋白質を有するF、HN 遺伝子欠損型ウイルスの作出も行った。また、VSV-G発現細胞を用いて、VSV-Gシュードタイプのウイルスを作製することにも成功した。これらの欠損型ウイルス産生技術は、パラミクソウイルスにおける遺伝子治療用ベクターの開発に道を開くものである。

PCTに基づいて公開される国際出願のパンフレット第一頁に掲載されたPCT加盟国を同定するために使用されるコード(参考情報)

AE	アラブ首長国連邦	DM	ドミニカ	KZ	カザフスタン	RU	ロシア
AG	アンティグア・バーブーダ	DZ	アルジェリア	LC	セントルシア	SD	スーダン
AL	アルバニア	EE	エストニア	LI	リヒテンシュタイン	SE	スウェーデン
AM	アルメニア	ES	スペイン	LK	スリ・ランカ	SG	シンガポール
AT	オーストリア	FI	フィンランド	LR	リベリア	SI	スロヴェニア
AU	オーストラリア	FR	フランス	LS	レソト	SK	スロヴァキア
AZ	アゼルバイジャン	GA	ガボン	LT	リトアニア	SL	シエラ・レオネ
BA	ボスニア・ヘルツェゴビナ	GB	英国	LU	ルクセンブルグ	SN	セネガル
BB	バルバドス	GD	グレナダ	LV	ラトヴィア	SZ	スワジランド
BE	ベルギー	GE	グルジア	MA	モロッコ	TD	チャード
BF	ブルキナ・ファソ	GH	ガーナ	MC	モナコ	TG	トーゴ
BG	ブルガリア	GM	ガンビア	MD	モルドヴァ	TJ	タジキスタン
BJ	ベナン	GN	ギニア	MG	マダガスカル	TM	トルクメニスタン
BR	ブラジル	GR	ギリシャ	MK	マケドニア旧ユーゴスラヴィア	TR	トルコ
BY	ベラルーシ	GW	ギニア・ビサウ		共和国	TT	トリニダード・トバゴ
CA	カナダ	HR	クロアチア	ML	マリ	TZ	タンザニア
CF	中央アフリカ	HU	ハンガリー	MN	モンゴル	UA	ウクライナ
CG	コンゴ	ID	インドネシア	MR	モーリタニア	UG	ウガンダ
CH	スイス	IE	アイルランド	MW	マラウイ	US	米国
CI	コートジボアール	IL	イスラエル	MX	メキシコ	UZ	ウズベキスタン
CM	カメルーン	IN	インド	MZ	モザンビーク	VN	ヴェトナム
CN	中国	IS	アイスランド	NE	ニジェール	YU	ユーゴスラヴィア
CR	コスタ・リカ	IT	イタリア	NL	オランダ	ZA	南アフリカ共和国
CU	キューバ	JP	日本	NO	ノルウェー	ZW	ジンバブエ
CY	キプロス	KE	ケニア	NZ	ニュージーランド		
CZ	チェッコ	KG	キルギスタン	PL	ポーランド		
DE	ドイツ	KP	北朝鮮	PT	ポルトガル		
DK	デンマーク	KR	韓国	RO	ルーマニア		

- 1 -

## 明細書

## エンベロープ遺伝子欠損パラミクソ科ウイルスベクター

技術分野

本発明は、エンベロープ遺伝子欠損型パラミクソウイルスベクターに関する。

背景技術

これまでの遺伝子治療の臨床研究のアプローチの多くはレトロウイルス、アデノウイルスおよびアデノ随伴ウイルスなどのウイルスベクターが利用されている。これらの遺伝子治療用ベクターは、導入効率および持続発現に制限があり、ベクター自体に細胞毒性及び免疫原性があるなど、医学応用上の大きな問題が存在する (Lamb, R.A. & Kolakofsky, D., Paramyxoviridae: the viruses and their replication. *in* Fields Virology, 3rd edn, (Edited by B.N. Fields, D.M. Knipe & P.P. Howley) pp.1177-1204 (Philadelphia, Lippincott-Raven. (1996))). これらの対策として新たなベクターがレンチウイルスやHSVをベースに提案されており、また既存ベクターの改良研究が精力的になされている。しかしながら、これらのベクターはいずれも生活環において、核内でDNAの形態で存在する。従って、患者の染色体とのランダムな相互作用に関わる安全性への危惧は完全に回避することは難しい。

最近のリバースジェネテックス技術の急速な進歩により、従来開発が遅れていたRNAウイルスをベースにしたベクターの開発が可能となりつつある。組み換えRNAウイルスは高い遺伝子導入効率と発現能力を示し、遺伝子治療用ベクターとしての高いポテンシャルティが示唆される (Roberts, A. & Rose, J., K., Virology 247, 1-6 (1998); Rose, J., Proc. Natl. Acad. Sci. USA 94, 14998-15000 (1996); Palese, P. et. al., Proc. Natl. Acad. Sci. USA 93, 11354-11358 (1996))。

- 2 -

しかし、実用的に利用可能な弱毒ウイルスの欠損型ゲノム由来のパラミクソウイルスベクターはまだ報告されていない。

マイナス鎖RNAをゲノムに持つパラミクソウイルスベクターは、レトロウイルス、DNAウイルス、またはプラス鎖RNAウイルスベクターとは大きく異なる幾つの特徴を持っている。そのゲノムまたはアンチゲノムは直接にmRNAとしては機能せず、ウイルスのタンパク質合成やゲノム複製を開始させることはできない。ウイルスのRNAゲノムもアンチゲノムも常にリボ核酸タンパク質複合体 (ribonucleoprotein complex; RNP) の形で存在し、プラス鎖RNAウイルスに見られるような、mRNAsが相補的な裸のゲノムRNAにハイブリダイズしてゲノムのRNPへのアセンブリを妨害するといったアンチセンスの問題が殆ど起きない。これらのウイルスは自身のRNAポリメラーゼを持っており、RNP複合体を鋳型にしてウイルスmRNAの転写またはウイルスゲノムの複製を行う。特筆すべきことにマイナス鎖RNA (nsRNA) ウイルスは宿主細胞の細胞質でのみ増殖し、DNAフェーズを持たないため染色体への組み込み (integration) は起こらない。更にはRNA同士の相同組み換えも認められていない。これらの性質はマイナス鎖RNAウイルスの遺伝子発現ベクターとしての安定性と安全性に大きく寄与するものと思われる。

本発明者らはネガティブ鎖RNAウイルスの中でもセンダイウイルス (Sendai virus; SeV) に注目してきた。SeVは非分節型マイナス鎖RNAウイルスで、パラミクソウイルス (paramyxovirus) に属し、murine parainfluenza virusの一種である。このウイルスは二つのエンベロープ糖タンパク質であるヘマグルチニン-ノイラミニダーゼ (hemagglutinin-neuraminidase; HN) とフュージョンタンパク質 (fusion protein; F) を介して宿主細胞膜に接着、膜融合を起こし、効率的に自分のRNAポリメラーゼとリボヌクレオプロテイン (RNP) 複合体の形で存在するRNAゲノムを細胞質に放出し、そこでウイルスのmRNAの転写及びゲノムの複製を行う (Bitzer, M. et al., J. Virol. 71(7):5481-5486, 1997)。ウイルスエンベロープ蛋白質Fは活性の無い前駆蛋白 ( $F_0$ ) として合成され、トリプシンによるタン



- 3 -

パク質分解 (proteolytic cleavage) で F1 と F2 に解裂され (Kido, H. et al., Biopolymers (Peptide Science) 51(1):79-86, 1999)、活性型蛋白質となり膜融合を引き起こす。このウイルスはヒトに対して病原性がないと言われている。また、ラボ弱毒株 (Z strain) も分離されており、自然宿主であるげっ歯類に対し軽度の肺炎を誘発する程度である。この株はパラミクソウイルスの転写複製機構等の分子レベルにおける研究モデルとして広く用いられており、またハイブリドーマの作製にも使われてきた。このような高い安全性の外にこのウイルスは細胞株または鶏卵で  $10^9 \sim 10^{11}$  pfu/ml という高い生産タイターを示す。最近成功したネガティブ鎖RNAウイルスベクターのcDNAからの回収システムの中で、センダイウイルスの場合は特に高い再構成効率を示している。外来遺伝子を導入した組み換え型野生ウイルスでは効率的且つ安定的に導入外来遺伝子を発現する能力が注目されている。

このように、マイナス鎖RNAウイルスは遺伝子導入ベクターとして多くの利点を有するが、遺伝子治療に応用するには、細胞に感染させた場合、感染性粒子が放出されない安全性の高いベクターを開発することが望まれる。このためには、野生型のウイルス産生能を欠損させた欠損型ウイルスを大量生産する技術が必要である。しかし、これまで応用可能なエンベロープ遺伝子欠損型ゲノムをベースにしたベクターの開発はまだ成功していない。

#### 発明の開示

本発明は、エンベロープ遺伝子を欠損したパラミクソウイルスベクターを提供することを課題とする。

伝播性を欠く、遺伝子治療により適したパラミクソウイルスベクターを構築するため、本発明者らは、SeVのF遺伝子をゲノム上から欠失させ、GFP遺伝子をレポーターとして導入したcDNAを用いて、センダイウイルスのF蛋白を発現する細胞株で感染性ウイルス粒子を回収する方法を確立した。このF遺伝子欠損ウイルスベ

クターは、初代培養したラットの神経細胞、primitiveなマウスの血液幹細胞、ヒトの正常細胞など多様な細胞に高い効率で遺伝子導入され、高い遺伝子発現を示した。さらに、in vivoではラットの脳に投与して高発現が得られた。このF遺伝子欠損型SeVベクターは感染した細胞で比較的に長く持続的にかつ強力に遺伝子を発現し、二次的に感染性ウイルス粒子を産生せず、隣接した細胞へも伝播しないため、遺伝子治療用ベクターとしての有用性が示唆された。

さらに本発明者らは、F遺伝子とHN遺伝子の両方を欠損させたSeVベクターcDNAを作製し、センダイウイルスのF蛋白およびHN蛋白質を発現する細胞株で感染性ウイルス粒子を回収する方法を確立した。また、このSeVベクターcDNAを、F発現細胞に導入することによって、HN蛋白質を欠失したSeVベクターを作製することにも成功した。

このように本発明は、マイナス鎖RNAウイルスを基本骨格とした実用化可能な新しいエンベロープ遺伝子欠損型ベクターシステムを初めて確立するものである。F遺伝子欠損、またはFHN遺伝子欠損ゲノムcDNAから、ヘルパー細胞を利用して感染性欠損ウイルス粒子の回収に成功したことは、センダイウイルスの優れた特徴を生かした新規な遺伝子治療用ベクターの研究開発に道を開くものである。

本発明の欠損型センダイウイルスベクターは遺伝子導入効率も広範な細胞種に対して極めて高く外来遺伝子を驚異的に発現する能力を持っている。さらに、感染細胞で持続的に発現し、2次的な感染性ウイルス粒子を放出しないことから、ウイルスの伝播性を完全に無くした安全性の高いベクターであることが判明した。

RNAウイルスではゲノムの安定性の問題が指摘され得るが、SeVベクターによる異種遺伝子発現の結果ではウイルスを連続多代継代しても殆ど塩基の変異が認められず、挿入異種遺伝子を長期間に渡って安定に発現する事が示されている (Yu, D. et al. Genes cells 2, 457-466 (1997))。このマイナス鎖RNAウイルスレプリコンをベースにしたベクターは、既に成功しているポジティブ鎖 (positive-

strand) RNAウイルスであるセムリキ森林ウイルス (Semliki forest virus) またはシンドビスウイルス (Sindbis viruses) のレプリコンをベースにしたウイルスベクターに比べ、ゲノムの安定性や、カプシド構造タンパク質を持たないことによる導入遺伝子のサイズまたはパッケージングの柔軟性 (flexibility) など性質上幾つかのメリットがある。野生型センダイウイルスベクターは、外来DNAを少なくとも 4 kbpまで導入可能であり、欠失型ベクターではさらに導入サイズが大きくなる。転写ユニットを付加することによって2種類以上の遺伝子を同時に発現する事が可能かもしれない。このセンダイウイルスのレプリコンをベースにしたベクターは理論的には細胞質で多コピーに複製されたRNPが細胞の分裂に伴い娘細胞にも分配されるため持続発現が期待される。実際ある種の血液系細胞ではin vitro実験によりそれが証明されている。さらに、本発明者等は、センダイウイルスベクターが血球系の細胞、特に顆粒球系細胞に高い効率で遺伝子導入され、c-kit陽性のprimitive細胞にも導入されることを確認していることから、このベクターは非常に広い組織適用範囲を持つ応用可能性の高いベクターになり得ることが示唆される。

即ち本発明は、エンベロープ遺伝子欠損センダイウイルスベクターに関し、より具体的には、

(1) (a) パラミクソ科ウイルスの少なくとも一つのエンベロープタンパク質を発現しないように改変されたパラミクソウイルスに由来する (一) 鎖一本鎖RNA、および (b) 該 (一) 鎖一本鎖RNAと結合するタンパク質、からなる複合体を含むパラミクソ科ウイルスベクター、

(2) (一) 鎖一本鎖RNAがNPタンパク質、Pタンパク質、およびLタンパク質を発現し、Fタンパク質および/またはHNタンパク質を発現しないように改変されている、(1) に記載のベクター、

(3) (一) 鎖一本鎖RNAから発現しないように改変されたエンベロープタンパク質の少なくとも一つを含む、(1) または (2) に記載のベクター、

- 6 -

- (4) VSV-Gタンパク質を含む、(1) から (3) のいずれかに記載のベクター、
- (5) (一) 鎖一本鎖RNAがセンダイウイルスに由来する、(1) から (4) のいずれかに記載のベクター、
- (6) (一) 鎖一本鎖RNAがさらに外来遺伝子をコードしている、(1) から (5) のいずれかに記載のベクター、
- (7) (1) から (6) のいずれかに記載のベクターに含まれる (一) 鎖一本鎖RNA またはその相補鎖をコードするDNA、
- (8) (1) から (6) のいずれかに記載のベクターの製造方法であって、
- (a) パラミクソ科ウイルスの少なくとも一つのエンベロープタンパク質を発現しないように改変されたパラミクソウイルスに由来する (一) 鎖一本鎖RNA またはその相補鎖をコードするベクターDNAを、エンベロープタンパク質を発現する細胞に導入して発現させる工程、および
- (b) 該細胞を培養し、その培養上清からウイルス粒子を回収する工程、を含む方法、
- (9) (1) から (6) のいずれかに記載のベクターの製造方法であって、
- (a) パラミクソ科ウイルスの少なくとも一つのエンベロープタンパク質を発現しないように改変されたパラミクソウイルスに由来する (一) 鎖一本鎖RNA、および該 (一) 鎖一本鎖RNAと結合するタンパク質、からなる複合体を、エンベロープタンパク質を発現する細胞に導入する工程、および
- (b) 該細胞を培養し、その培養上清からウイルス粒子を回収する工程、を含む方法、
- (10) 工程 (b) における細胞の培養が、エンベロープタンパク質を発現する細胞との共培養である、(8) または (9) に記載の方法、
- (11) 工程 (b) における細胞の培養において、該細胞に、エンベロープタンパク質を発現する細胞を重層して培養を行う、(8) または (9) に記載の方法、
- (12) 細胞が発現するエンベロープタンパク質の少なくとも1つが、前記 (一)

- 7 -

)鎖一本鎖RNAから発現しないように改変されたエンベロープタンパク質の少なくとも1つと同一である、(8)から(11)のいずれかに記載の方法、

(13)細胞が発現するエンベロープタンパク質の少なくとも1つがVSV-Gタンパク質である、(8)から(12)のいずれかに記載の方法、に関する。

なお、本発明において「ベクター」とは、宿主内で外来遺伝子を発現させるための核酸分子をパッケージングしたウイルス粒子を指す。

また、パラミクソウイルス科ウイルスの「NP、P、M、F、HN、およびL遺伝子」とは、それぞれヌクレオキャプシド、ホスホ、マトリックス、フュージョン、ヘマグルチニン-ノイラミニダーゼ、およびラージ蛋白質をコードする遺伝子のことを指す。パラミクソウイルス亜科に属する各ウイルスにおける各遺伝子は、一般に次のように表記される。また、一般に、NP遺伝子は「N遺伝子」と表記されることもある。

レスピロウイルス属	N	P/C/V	M	F	HN	-	L
ルブラウイルス属	N	P/V	M	F	HN (SH)		L
モービリウイルス属	N	P/C/V	M	F	H	-	L

例えばパラミクソウイルス科 (Paramyxoviridae) のレスピロウイルス (Respirovirus) に分類されるセンダイウイルスの各遺伝子の塩基配列のデータベースのアクセッション番号は、NP遺伝子については M29343、M30202、M30203、M30204、M51331、M55565、M69046、X17218、P遺伝子については M30202、M30203、M30204、M55565、M69046、X00583、X17007、X17008、M遺伝子については D11446、K02742、M30202、M30203、M30204、M69046、U31956、X00584、X53056、F遺伝子については D00152、D11446、D17334、D17335、M30202、M30203、M30204、M69046、X00152、X02131、HN遺伝子については D26475、M12397、M30202、M30203、M30204、M69046、X00586、X02808、X56131、L遺伝子については D00053、M30202、M30203、M30204、M69040、X00587、X58886を参照のこと。

本発明は、エンベロープ遺伝子欠損型のパラミクソ科ウイルスベクターに関する

る。該ウイルスベクターは、少なくとも一つのエンベローブタンパク質を発現しないように改変されたパラミクソウイルスに由来する（－）鎖一本鎖RNAを含むことを特徴とする。パラミクソウイルスは、一般に、エンベローブの内部にRNAとタンパク質からなる複合体（リボヌクレオプロテイン；RNP）を含んでいる。RNPに含まれるRNAはパラミクソウイルスのゲノムである（－）鎖（ネガティブ鎖）の一本鎖RNAであり、タンパク質はこのRNAに結合して複合体を形成している。本発明のパラミクソ科ウイルスベクターは、（a）パラミクソ科ウイルスの少なくとも一つのエンベローブタンパク質を発現しないように改変されたパラミクソウイルスに由来する（－）鎖一本鎖RNA、および（b）該（－）鎖一本鎖RNAと結合するタンパク質、からなる複合体をそのウイルス粒子内に含んでいる。（－）鎖一本鎖RNAと結合するタンパク質とは、該（－）鎖一本鎖RNAと直接および/または間接に結合し、該（－）鎖一本鎖RNAと複合体を形成するタンパク質のことを言う。一般に、パラミクソウイルスの（－）鎖一本鎖RNA（ゲノムRNA）には、NPタンパク質、Pタンパク質、およびLタンパク質が結合している。このRNPに含まれるRNAがウイルスゲノムの転写および複製のための鋳型となる（Lamb, R.A., and D. Kolakofsky, 1996, *Paramyxoviridae: The viruses and their replication*. pp.1177-1204. In *Fields Virology*, 3rd edn. Fields, B. N., D. M. Knipe, and P. M. Howley et al. (ed.), Raven Press, New York, N. Y.）。本発明の複合体には、パラミクソウイルスに由来する（－）鎖一本鎖RNAおよびそれに結合するパラミクソウイルスに由来するタンパク質からなる複合体が含まれる。本発明のベクターは、例えばパラミクソウイルスの（－）鎖一本鎖RNAにこれらのタンパク質（NP、P、およびLタンパク質）が結合したRNPを含むものである。一般に、パラミクソウイルスのRNP複合体は、細胞内で自立的にRNP複合体を複製する能力を有する。このように、細胞に導入されたベクターは細胞内でRNPを増幅させることにより、遺伝子（複合体に含まれるRNA）のコピー数を増やす。これにより、外来遺伝子を持つベクターからの外来遺伝子の高い発現がもたらされる。本発明のベクターは、好ましくは、

細胞内で複合体（RNP）に含まれるRNAを複製する能力を有するものである。

本発明を適用可能なパラミクソ科ウイルスとしては、センダイウイルスに加え、例えば、麻疹ウイルス、サルパラインフルエンザウイルス（SV5）、ヒトパラインフルエンザウイルス3型が挙げられるが、これらに制限されない。

ウイルスベクター中の（－）鎖一本鎖RNAは、典型的には、NPタンパク質、Pタンパク質、およびLタンパク質を発現し、Fタンパク質および/またはHNタンパク質を発現しないように改変されている。

センダイウイルス（Sendai virus; SeV）の場合、天然のウイルスのゲノムサイズは約15,000塩基で、ネガティブ鎖は 3' の短いリーダー領域に続き、NP（ヌcleoキャプシド）、P（ホスホ）、M（マトリックス）、F（フュージョン）、HN（ヘマグルチニン-ノイラミニダーゼ）、およびL（ラージ）蛋白質をコードする6つの遺伝子が並んでおり、短い5' トレイラー領域を他端に有する。本発明においては、このうち F、HN、および M 遺伝子のうちいずれか、あるいはそれらの組み合わせを欠損するゲノムを設計することにより、エンベロープタンパク質を発現しないように改変することができる。好ましくはF遺伝子またはHN遺伝子、あるいはF遺伝子とHN遺伝子の両方を欠損している。RNPの形成にはこれらの蛋白質は必要ないため、NP、P、およびLタンパク質の存在下でこのゲノムRNA（ポジティブ鎖またはネガティブ鎖）を転写させることにより、本発明のRNPを製造することができる。RNPの形成は、例えばLLC-MK2細胞などで行わせることができる。NP、P、およびLタンパク質の供給は、各遺伝子をコードする発現ベクターを細胞に導入することにより行われ得る（実施例参照）。また、各遺伝子は宿主細胞の染色体に組み込まれていてもよい。RNPを形成させるために発現させる NP、P、およびL遺伝子は、ベクターのゲノムにコードされる NP、P、およびL遺伝子と完全に同一である必要はない。すなわち、これらの遺伝子がコードする蛋白質のアミノ酸配列は、RNPゲノムがコードするタンパク質のアミノ酸配列そのままでもなくとも、ゲノムRNAと結合し、細胞内でRNPの複製を行う活性を持つ限り、変異を導入したり、あるいは他のウ

- 10 -

イルスの相同遺伝子で代用してもよい。一端RNPが形成されれば、このRNPから NP、P、およびL遺伝子が発現され、細胞内で自立的にRNPが複製し、ウイルスベクターが生産される。

細胞内でベクターを再構成させる時にエンベローブタンパク質を細胞に発現させれば、このエンベローブタンパク質がウイルスベクターに取り込まれ、エンベローブタンパク質による感染性を保持するウイルスベクターを生産することができる。このようなベクターは、一度細胞に感染すると、細胞内でRNPを増殖させることはできても、それ自身はエンベローブ遺伝子を持たないため、初めと同じようなエンベローブタンパク質を持つウイルスを再度生産することはできない。このようなベクターは、特に遺伝子治療など高い安全性が要求される分野に極めて有用である。

ウイルス再構成の際に、(一)鎖一本鎖RNAにおいて発現しないように改変されたエンベローブタンパク質、すなわちゲノムにおいて欠損させたエンベローブ遺伝子を発現させれば、野生型ウイルスと同等の感染性を有するウイルスベクターを製造することができる。また、ゲノムにおいて欠損させたエンベローブ遺伝子の一部を発現させることも考えられる。例えば、F遺伝子とHN遺伝子の両方を欠損するゲノムに対してFタンパク質のみを発現させると、Fタンパク質をエンベローブに有するウイルスベクターが生産される。HNタンパク質を持たずFタンパク質のみを持つウイルスは、アシアロ糖蛋白リセプター(ASG-R)を介して肝臓の細胞に特異的に感染するベクターとして利用され得る。このように、(一)鎖一本鎖RNAにおいて発現しないように改変されたエンベローブタンパク質の少なくとも1つを含むパラミクソ科ウイルスベクターも、本発明に含まれる。

また、(一)鎖一本鎖RNAにおいて発現しないように改変されたエンベローブタンパク質とは異なるエンベローブタンパク質を用いて本発明のベクターを再構成させることも可能である。このようなエンベローブタンパク質に特に制限はない。例えば、他のウイルスのエンベローブタンパク質、例えば水疱性口内炎ウイル



ス (VSV) のGタンパク質 (VSV-G) を挙げることができる。本発明のパラミクソ科ウイルスベクターは、VSV-Gタンパク質などのように、ゲノムが由来するウイルス以外のウイルスに由来するエンベロープタンパク質を含むシュードタイプウイルスベクターが含まれる。

本発明のウイルスベクターは、通常、(a) パラミクソ科ウイルスの少なくとも一つのエンベロープタンパク質を発現しないように改変されたパラミクソウイルスに由来する (一) 鎖一本鎖RNAまたはその相補鎖をコードするベクターDNAを、エンベロープタンパク質を発現する細胞 (ヘルパー細胞) に導入して発現させ、(b) 該細胞を培養し、その培養上清からウイルス粒子を回収することにより調製することができる。ベクターDNAを発現させる時に、NP、L、およびPタンパク質を共発現させておくことでRNPが形成され、エンベロープタンパク質を持つウイルスが構築される。

ヘルパー細胞で発現させるベクターDNAは、本発明のベクターに含まれる (一) 鎖一本鎖RNA (ネガティブ鎖) またはその相補鎖 (ポジティブ鎖) をコードしている。例えば、(一) 鎖一本鎖RNAまたはその相補鎖をコードするDNAをT7プロモーターの下流に連結させ、T7 RNAポリメラーゼによりRNAに転写させる。ベクターDNAは、大腸菌で増幅できるようにプラスミドにクローニングされていてもよい。細胞内で転写させる鎖は、ウイルスのポジティブ鎖でもネガティブ鎖でもよいが、ポジティブ鎖が転写されるようにすることが再構成の効率を上げるためには好ましい。

ヘルパー細胞としては、エンベロープタンパク質を発現する細胞が用いられる。上記のように、ヘルパー細胞は、ウイルスベクターにおいて欠損している全てのエンベロープ遺伝子のタンパク質を発現する細胞に限定されず、例えば、F、HN遺伝子欠損センダイウイルスベクターDNAに対しては、Fタンパク質のみを発現する細胞をヘルパー細胞として用いることもできる。また、該ウイルスベクターにおいて欠損しているエンベロープ遺伝子がコードするタンパク質とは異なるエン

ペローブタンパク質を発現する細胞でありうる。例えば、上記のようにエンペローブタンパク質として、パラミクソ科ウイルスのエンペローブタンパク質ではないエンペローブタンパク質（例えば、VSV-Gタンパク質）を用いることも可能である。

例えば、エンペローブ遺伝子が欠損した組換えセンダイウイルスベクターゲノムを発現するプラスミドを、欠損したエンペローブ蛋白質を発現するベクターならびに、NP、P/CおよびL蛋白質の発現ベクターと共に宿主細胞にトランスフェクションすることにより、ウイルスベクターの再構成を行うことができる。また、例えば、F遺伝子が染色体に組込まれた宿主細胞を用いて製造することもできる。ウイルスゲノム以外から供給されるこれらの蛋白質群は、そのアミノ酸配列はウイルス由来の配列そのままでもなくとも、核酸の導入における活性が天然型のそれと同等かそれ以上ならば、変異を導入したり、あるいは他のウイルスの相同遺伝子で代用してもよい。一般に、エンペローブタンパク質は細胞毒性を示すものが多いため、誘導性プロモーターの制御下にベクターの再構成時にのみ発現させることもできる（実施例参照）。

RNPまたはウイルスが形成されれば、このRNPまたはウイルスを上記のヘルパー細胞に再度導入して培養することにより、本発明のベクターを増幅することができる。この過程は、(a) パラミクソ科ウイルスの少なくとも一つのエンペローブタンパク質を発現しないように改変されたパラミクソウイルスに由来する（-）鎖一本鎖RNA、および該（-）鎖一本鎖RNAと結合するタンパク質、からなる複合体を、エンペローブタンパク質を発現する細胞に導入する工程、および（b）該細胞を培養し、その培養上清からウイルス粒子を回収する工程、を含む。

RNPを細胞に導入するには、例えばリポフェクトアミンやポリカチオニックリポソームなどと共に複合体を形成させて導入することが可能である。具体的には、種々のトランスフェクション試薬が利用できる。例えば、DOTMA (Boehringer)、Superfect (QIAGEN #301305)、DOTAP、DOPE、DOSPER (Boehringer #1811169) な

どが挙げられる。エンドソーム中での分解を防ぐため、クロロキンを加えることもできる (Calos, M.P., 1983, Proc. Natl. Acad. Sci. USA 80: 3015)。

上記のようにして細胞においてウイルスベクターが構築されれば、この細胞を、エンベロープタンパク質を発現する細胞と共培養することにより、ウイルスベクターをさらに増幅することができる。このような方法としては、例えば実施例 1 2 に記載したように、ウイルスを産生する細胞にエンベロープタンパク質を発現する細胞を重層する方法が好適である。

エンベロープタンパク質としては、ウイルスのエンベロープタンパク質以外にも、例えば、特定の細胞に接着しうるような、接着因子、リガンド、受容体等由来のポリペプチドを細胞外領域に有し、ウイルスエンベロープ由来のポリペプチドを細胞内領域に有するキメラタンパク質などを用いることが可能である。これにより、特定の組織を標的とするベクターを作り出すこともできる。また、本発明のウイルスベクターは、例えば、免疫原性を低下させるために、または、RNA の転写効率や複製効率を高めるために、ベクターに含まれるウイルス遺伝子が改変されたものであってもよい。

本発明のウイルスベクターは、(一) 鎖一本鎖RNA中に外来遺伝子をコードするRNAを含みうる。外来遺伝子としては、標的細胞中で発現させたい所望の遺伝子を用いることが可能である。例えば、遺伝子治療などを目的とする場合には、該ウイルスベクターDNAに対象となる疾患の治療用遺伝子を挿入する。ウイルスベクターDNAに外来遺伝子を導入する場合は、例えば、センダイウイルスベクターDNAにおいては、転写終結(E)配列と転写開始(S)配列との間などに、6の倍数の塩基数を有する配列を挿入することが望ましい (Journal of Virology, Vol.67, No.8, 1993, p.4822-4830)。外来遺伝子は、ウイルスの各遺伝子 (NP、P、M、F、HN、およびL遺伝子) の前または後ろに挿入することができる (実施例参照)。前後の遺伝子の発現を妨げないようにするため、外来遺伝子の前または後ろに適宜 E-I-S 配列 (転写開始配列-介在配列-転写終結配列) またはその部分を挿入する。挿

入した外来性遺伝子の発現量は、外来遺伝子上流に付加する転写開始配列の種類により調節することができる。また、遺伝子挿入の位置、また遺伝子の前後の塩基配列により調節しうる。例えば、センダイウイルスにおいては、挿入位置が（－）鎖RNAの3'端に近いほど（野生型ウイルスのゲノム上の遺伝子配置においては、NP遺伝子に近いほど）、挿入された遺伝子の発現量が高い。外来遺伝子の高い発現を得るためには、外来遺伝子をNP遺伝子上流（マイナス鎖においては3'側）またはNP遺伝子とP遺伝子の間など、ネガティブ鎖ゲノムにおいて上流領域に挿入することが好ましい。逆に、挿入位置がネガティブ鎖RNAの5'端に近いほど（野生型ウイルスのゲノム上の遺伝子配置においては、L遺伝子に近いほど）、挿入された遺伝子の発現量が低くなる。外来遺伝子の発現を低く抑えるためには、例えばネガティブ鎖の最も5'側、すなわち野生型ウイルスゲノムにおいてはL遺伝子の下流（ネガティブ鎖においてはL遺伝子の5'隣接部位）、またはL遺伝子上流（ネガティブ鎖においてはL遺伝子の3'隣接部位）に外来遺伝子を挿入する。外来遺伝子を容易に挿入できるようにするために、挿入部位にクローニングサイトを設計することができる。クローニングサイトは、例えば制限酵素の認識配列とすることができる。ゲノムをコードするベクターDNA中の当該制限酵素部位に外来遺伝子断片を挿入することができる。クローニングサイトは、複数の制限酵素認識配列を有する、いわゆるマルチクローニングサイトとしてもよい。本発明のベクターは、このように挿入した以外に位置に他の外来遺伝子を保持していてもよい。

外来遺伝子を有する組換えセンダイウイルスベクターは、例えば、Kato, A. et al., 1997, EMBO J. 16: 578-587及びYu, D. et al., 1997, Genes Cells 2: 457-466の記載に準じて、次のようにして構築することができる。

まず、所望の外来遺伝子のcDNA塩基配列を含むDNA試料を用意する。DNA試料は、25ng/ $\mu$ l以上の濃度で電気泳動的に単一のプラスミドと確認できることが好ましい。以下、外来遺伝子をNotI部位を利用してウイルスゲノムをコードするDNAに挿入する場合を例にとって説明する。目的とするcDNA塩基配列の中にNotI認識

部位が含まれる場合は、部位特異的変異挿入法などを用いて、コードするアミノ酸配列を変化させないように塩基配列を改変し、NotI部位を予め除去しておくことが好ましい。この試料から所望の遺伝子断片をPCRにより増幅回収する。増幅された断片の両端がNotI部位とし、さらに一端にセンダイウイルスの転写終結配列（E）、介在配列（I）及び転写開始配列（S）（E I S配列）のコピーを付加するために、NotI制限酵素切断部位配列及び転写終結配列（E）、介在配列（I）及び転写開始配列（S）と目的遺伝子の一部の配列を含むプライマー対として、フォワード側合成DNA配列及びリバース側合成DNA配列（アンチセンス鎖）を作成する。

例えば、フォワード側合成DNA配列は、NotIによる切断を保証するために 5' 側に任意の 2 以上のヌクレオチド（好ましくはGCG、GCCのNotI認識部位由来の配列が含まれない 4 塩基、更に好ましくはACTT）を選択し、その3' 側にNotI認識部位gcggccgcを付加し、さらにその3' 側にスペーサー配列として任意の 9 塩基または 9 に 6 の倍数を加えた数の塩基を付加し、さらにその3' 側に所望のcDNAの開始コドンATGからこれを含めてORFの約25塩基相当の配列を付加した形態とする。最後の塩基はGまたはCとなるように該所望のcDNAから約25塩基を選択してフォワード側合成オリゴDNAの3' の末端とすることが好ましい。

リバース側合成DNA配列は5' 側から任意の 2 以上のヌクレオチド（好ましくはGCG、GCCのNotI認識部位由来の配列が含まれない 4 塩基、更に好ましくはACTT）を選択し、その3' 側にNotI認識部位gcggccgcを付加し、さらにその3' 側に長さを調節するための挿入断片のオリゴDNAを付加する。このオリゴDNAの長さは、NotI認識部位gcggccgcを含め、cDNAの相補鎖塩基配列と後述するセンダイウイルスに由来するセンダイウイルスゲノムの E I S 塩基配列の合計が 6 の倍数になるように塩基数を設計する（いわゆる「6のルール (rule of six)」; Kolakofski, D. et al., J. Virol. 72:891-899, 1998）。さらに挿入断片の3' 側にセンダイウイルスの S 配列の相補鎖配列、好ましくは5'-CTTTCACCT-3'、I 配列、好ましくは5'-

AAG-3'、E配列の相補鎖配列、好ましくは5'-TTTTTCTTACTACGG-3'、さらにその3'側に所望のcDNA配列の終始コドンから逆に数えて約25塩基相当の相補鎖の最後の塩基がGまたはCになるように長さを選択して配列を付加し、リバーズ側合成オリゴDNAの3'の末端とする。

PCRは、例えば、ExTaqポリメラーゼ（宝酒造）を用いる通常の方法を用いることができる。好ましくはVentポリメラーゼ（NEB）を用いて行い、増幅した目的断片はNotIで消化した後、プラスミドベクターpBluescriptのNotI部位に挿入する。得られたPCR産物の塩基配列をシーケンサーで確認し、正しい配列のプラスミドを選択する。このプラスミドから挿入断片をNotIで切り出し、エンベロープ遺伝子を欠損するゲノムcDNAを含むプラスミドのNotI部位にクローニングする。またプラスミドベクターpBluescriptを介さずにNotI部位に直接挿入し、組換えセンダイウイルスcDNAを得ることも可能である。

本発明のウイルスベクターDNAは、これを試験管内または細胞内で転写させ、ウイルスのL、P、NPタンパク質により、RNPを再構成させ、このRNPを含むウイルスベクターを生成させることができる。ウイルスベクターDNAからのウイルスの再構成は、エンベロープタンパク質を発現する細胞を用いて、公知の方法に従って行うことができる（国際公開97/16539号；国際公開97/16538号；Durbin, A.P. et al., 1997, *Virology* 235: 323-332；Whelan, S.P. et al., 1995, *Proc. Natl. Acad. Sci. USA* 92: 8388-8392；Schnell, M.J. et al., 1994, *EMBO J.* 13: 4195-4203；Radecke, F. et al., 1995, *EMBO J.* 14: 5773-5784；Lawson, N.D. et al., *Proc. Natl. Acad. Sci. USA* 92: 4477-4481；Garcin, D. et al., 1995, *EMBO J.* 14: 6087-6094；Kato, A. et al., 1996, *Genes Cells* 1: 569-579；Baron, M.D. and Barrett, T., 1997, *J. Virol.* 71: 1265-1271；Bridgen, A. and Elliott, R.M., 1996, *Proc. Natl. Acad. Sci. USA* 93: 15400-15404）。ウイルスベクターDNAにおいて、F遺伝子、HN遺伝子、および/またはM遺伝子を欠失させた場合には、そのままでは感染性のウイルス粒子を形成しないが、宿主細胞に、これら欠失させた

遺伝子や他のウイルスのエンベロープ蛋白質をコードする遺伝子などを別途、導入し発現させることにより、感染性のウイルス粒子を形成させることが可能である。

ウイルスベクターDNAを細胞内に導入する方法には、次のような方法、①目的の細胞が取り込めるようなDNA沈殿物を作る方法、②目的の細胞による取りこみに適し、かつ細胞毒性の少ない陽電荷特性を持つDNAを含む複合体を作る方法、③目的の細胞膜に、DNA分子が通り抜けられるだけに十分な穴を電気パルスによって瞬間的に開ける方法などがある。

②としては、種々のトランスフェクション試薬が利用できる。例えば、DOTMA (Boehringer)、Superfect (QIAGEN #301305)、DOTAP、DOPE、DOSPER (Boehringer #1811169) などが挙げられる。①としては例えばリン酸カルシウムを用いたトランスフェクション法が挙げられ、この方法によって細胞内に入ったDNAは貪食小胞に取り込まれるが、核内にも十分な量のDNAが入ることが知られている (Graham, F.L. and Van Der Eb, J., 1973, Virology 52: 456; Wigler, M. and Silverstein, S., 1977, Cell 11: 223)。ChenおよびOkayamaはトランスファー技術の最適化を検討し、1) 細胞を共沈殿物のインキュベーション条件を 2~4% CO<sub>2</sub>、35℃、15~24時間、2) DNAは直鎖状より環状のものが活性が高く、3) 沈殿混液中のDNA濃度が 20~30 µg/ml のとき最適な沈殿が得られると報告している (Chen, C. and Okayama, H., 1987, Mol. Cell. Biol. 7: 2745)。②の方法は、一過的なトランスフェクションに適している。古くはDEAE-デキストラン (Sigma #D-9885 M.W. 5 × 10<sup>5</sup>) 混液を所望のDNA濃度比で調製し、トランスフェクションを行う方法が知られている。複合体の多くはエンドソームの中で分解されてしまうため、効果を高めるためにクロロキンを加えることもできる (Calos, M.P., 1983, Proc. Natl. Acad. Sci. USA 80: 3015)。③の方法は電気穿孔法と呼ばれる方法で、細胞選択性がないという点で①や②の方法に比べて汎用性が高い。効率はパルス電流の持続時間、パルスの形、電界 (電極間のギャップ、電圧) の強さ、バッファーの導

電率、DNA濃度、細胞密度の最適条件下で良いとされている。

以上、3つのカテゴリーの中で②の方法は操作が簡便で多量の細胞を用いて多数の検体を検討することができるので、本発明においては、トランスフェクション試薬が適している。好適には Superfect Transfection Reagent (QIAGEN, Cat No. 301305)、または DOSPER Liposomal Transfection Reagent (Boehringer Mannheim, Cat No. 1811169) が用いられる。

cDNAからの再構成は具体的には次のようにして行うことができる。

24穴から6穴程度のプラスチックプレートまたは100mmペトリ皿上で、10%ウシ胎児血清(FCS)および抗生物質 (100 units/ml ペニシリンGおよび100 $\mu$ g/ml ストレプトマイシン) を含む最少必須培地 (MEM)を用いてサル腎臓由来細胞株LLC-MK2を70~80%コンフルエントになるまで培養し、例えば 1 $\mu$ g/ml psoralen (ソラレン) 存在下 UV照射処理を20分処理で不活化した、T7ポリメラーゼを発現する組換えワクシニアウイルスvTF7-3 (Fuerst, T.R. et al., Proc. Natl. Acad. Sci. USA 83: 8122-8126, 1986、Kato, A. et al., Genes Cells 1: 569-579, 1996) を2 PFU/細胞で感染させる。ソラレンの添加量およびUV照射時間が適宜調整することができる。感染1時間後、2~60 $\mu$ g、より好ましくは3~5 $\mu$ gの上記の組換えセンダイウイルスcDNAを、全長センダイウイルスゲノムの生成に必須なトランスに作用するウイルスタンパク質を発現するプラスミド (24-0.5 $\mu$ gのpGEM-N、12-0.25 $\mu$ gのpGEM-P、および24-0.5 $\mu$ gのpGEM-L、より好ましくは1 $\mu$ gのpGEM-N、0.5 $\mu$ gのpGEM-P、および1 $\mu$ gのpGEM-L) (Kato, A. et al., Genes Cells 1: 569-579, 1996) と共にSuperfect (QIAGEN社) を用いたリポフェクション法等によりトランスフェクションする。トランスフェクションを行った細胞は、所望により100 $\mu$ g/mlのリファンピシン (Sigma) 及びシトシンアラビノシド (AraC)、より好ましくは40 $\mu$ g/mlのシトシンアラビノシド (AraC) (Sigma) のみを含む血清不含のMEMで培養し、ワクシニアウイルスによる細胞毒性を最少にとどめ、ウイルスの回収率を最大にするように薬剤の最適濃度を設定する(Kato, A. et al., 1996, Genes Cells



1: 569-579)。トランスフェクションから48～72時間程度培養後、細胞を回収し、凍結融解を3回繰り返して細胞を破碎した後、エンベロープタンパク質を発現するLLC-MK2細胞にトランスフェクションして培養する。培養3～7日後に培養液を回収する。あるいは、NP、L、P発現プラスミドを初めからエンベロープタンパク質を発現するLLC-MK2細胞にトランスフェクションするか、またはエンベロープ発現プラスミドを共にトランスフェクションすれば、感染性ウイルスベクターをより効率良く得ることができる。この細胞は、エンベロープタンパク質を発現するLLC-MK2細胞に重層して培養することによってウイルスベクターを増幅することができる(実施例参照)。培養上清に含まれるウイルス力価は赤血球凝集活性(HA)を測定することにより決定することができる。HAは「endo-point 希釈法」(Kato, A. et al., 1996, Genes Cells 1: 569-579)により決定することができる。得られたウイルスストックは-80℃で保存することができる。

本発明の組換えセンダイウイルスベクターは、例えば生理食塩水やリン酸緩衝生理食塩水(PBS)などで適宜希釈して組成物とすることができる。本発明の組換えセンダイウイルスベクターを鶏卵で増殖させた場合等においては漿尿液を含むこともできる。本発明の組換えセンダイウイルスベクター含有組成物には、脱イオン水、5%デキストロース水溶液等の生理学的に許容しうる媒体を含んでいてもよい。さらに、その他にも、安定剤、殺生物剤等が含有されていてもよい。

ウイルスベクターが再構成する限り、再構成に用いる宿主細胞は特に制限されない。例えば、センダイウイルスベクターの再構成においては、サル腎由来のCV-1細胞やLLC-MK2細胞、ハムスター腎由来のBHK細胞などの培養細胞を使うことができる。これらの細胞に適当なエンベロープタンパク質を発現させることで、そのエンベロープを有する感染性ウイルス粒子を得ることもできる。また、大量にセンダイウイルスベクターを得るために、例えばエンベロープ遺伝子を発現するベクターと共に上記の宿主から得られたウイルスベクターを発育鶏卵に感染させ、該ベクターを増幅することができる。または、エンベロープタンパク質遺伝子が

組み込まれたトランスジェニック鶏卵を用いてウイルスベクターを生産することも可能である。鶏卵を使ったウイルスベクターの製造方法は既に開発されている（中西ら編, (1993), 「神経科学研究の先端技術プロトコールIII, 分子神経細胞生理学」, 厚生社, 大阪, pp.153-172）。具体的には、例えば、受精卵を培養器に入れ9～12日間 37～38℃で培養し、胚を成長させる。エンベローブタンパク質を発現するベクターと共にセンダイウイルスベクターを漿尿膜腔へ接種し、数日間卵を培養してウイルスベクターを増殖させる。培養期間等の条件は、使用する組換えセンダイウイルスにより変わり得る。その後、ウイルスを含んだ漿尿液を回収する。漿尿液からのセンダイウイルスベクターの分離・精製は常法に従って行うことができる（田代真人, 「ウイルス実験プロトコール」, 永井、石浜監修, メジカルビュー社, pp.68-73, (1995)）。

エンベローブタンパク質を発現するベクターとして、本発明のウイルスベクター自体を用いることが考えられる。例えば、ゲノム上で欠損しているエンベローブ遺伝子が異なる2種のベクターを同じ細胞に導入すれば、それぞれで欠損するエンベローブタンパク質が、もう一方の複合体からの発現により供給されるため、互いに相補しあって感染力のあるウイルス粒子が形成され、複製サイクルがまわりウイルスベクターが増幅される。すなわち、2種またはそれ以上の本発明のベクターを、エンベローブタンパク質を相補する組み合わせで接種すれば、それぞれのエンベローブ遺伝子欠損型ウイルスベクターの混合物を大量かつ低コストで生産することができる。このようにして生産された混合ウイルスは、ワクチン等にも有用である。また、これらのウイルスは、エンベローブ遺伝子が欠損している分、エンベローブ遺伝子を欠損していないウイルスに比べゲノムサイズが小さくなり、長い外来遺伝子を保持することができる。また、元々感染性のないこれらのウイルスは細胞外で希釈され共感染の維持が困難であることから、不稔化するため、環境放出管理上の利点がある。

外来遺伝子として疾患の治療用遺伝子を用いてウイルスベクターを調製すれば

、このベクターを投与して遺伝子治療を行なうことが可能となる。本発明のウイルスベクターの遺伝子治療への応用としては、直接投与による遺伝子発現、間接 (ex vivo) 投与による遺伝子発現のいずれの方法によっても、治療効果を期待できる外来遺伝子もしくは患者の体内で供給が不足している内在遺伝子等を発現させることが可能である。外来遺伝子としては特に制限はなく、蛋白質をコードする核酸に加え、例えば、アンチセンスまたはリボザイムなどのタンパク質をコードしない核酸であってもよい。また、外来遺伝子として、感染症に関する細菌またはウイルスの抗原をコードする遺伝子を用いれば、これを動物に投与することにより、該動物において免疫を誘導することができる。即ち、ワクチンとして利用することができる。

ワクチンとして用いる場合、例えば腫瘍、感染症、およびその他の一般的な疾患に対し本発明のウイルスベクターを適用することが考えられる。例えば腫瘍治療としては、腫瘍細胞、またはDC細胞などの抗原提示細胞 (APC) に本発明のベクターを用いて治療効果を有する遺伝子を発現させることができる。このような遺伝子としては、癌抗原 Muc-1 または Muc-1様ムチンタンデムリピートペプチド (米国特許第 5,744,144号)、メラノーマ gp100抗原などが挙げられる。このような遺伝子による治療は、乳癌、結腸癌、膵臓癌、前立腺癌、肺癌等、幅広い応用が示されている。また、アジュバント効果を高めるサイトカイン類を組み合わせることも有効である。このような遺伝子としては、例えば i) IL-2と一本鎖IL-12 との組み合わせ (Proc. Natl. Acad. Sci. USA 96 (15): 8591-8596, 1999)、ii) IL-2とインターフェロン- $\gamma$  (米国特許第 5,798,100号)、iii) 単独で用いられる顆粒球コロニー刺激因子 (GM-CSF)、iv) 脳腫瘍を治療対象とした GM-CSF と IL-4 の組み合わせ (J. Neurosurgery 90 (6), 1115-1124 (1999)) などが挙げられる。

感染症の治療としては、インフルエンザにおいては、例えば強毒株 H5N1 型エンベロープ、日本脳炎においては、例えばエンベロープキメラ (Vaccine, vol. 17,

No. 15-16, 1869-1882 (1999))、エイズにおいては、例えばHIV gagまたは SIV gag タンパク質 (J. Immunology (2000) vol. 164, 4968-4978)、HIVエンベロープタンパク質の経口投与による鎖クチン治療、ポリ乳酸-グリコール共重合体に包んでの投与 (Kaneko, H. et al., Virology 267: 8-16 (2000))、コレラにおいては、例えばコレラ毒素のBサブユニット (CTB) (Arakawa T, et al., Nature Biotechnology (1998) 16(10): 934-8, Arakawa T, et al., Nature Biotechnology (1998) 16(3): 292-7)、狂犬病においては、例えば狂犬病ウイルスの糖タンパク (Lodmell DL et al., 1998, Nature Medicine 4(8):949-52)、子宮頸癌においては、ヒトパピローマウイルス6型のカプシドタンパクL1 (J. Med. Virol, 60, 200-204 (2000)) などが挙げられる。

また、一般病への適用も考えられる。糖尿病においては、例えばI型糖尿病モデル動物において、インシュリン断片のペプチドの発現が行われている (Coon, B. et al., J. Clin. Invest., 1999, 104(2):189-94)。

#### 図面の簡単な説明

図1は、Cre-loxP誘導発現系によるF蛋白質の発現を解析したウエスタンブロット解析の結果を示す写真である。化学発光法により抗SeV-F抗体と交叉のみられる該転写膜上の蛋白質の検出を行った結果を示す。

図2は、Cre-loxP系により発現を誘導したF蛋白質の細胞表面へのディスプレイを解析した結果を示す図である。抗SeV-F抗体を用いてLLC-MK2/F7のフローサイトメトリー解析を行った結果を示す。

図3は、発現されたF蛋白質のトリプシンによる解裂をウエスタンブロット法により確認した結果を示す写真である。

図4は、細胞表面におけるHNの発現を赤血球の細胞表面への吸着実験で確認した結果を示す写真である。

図5は、欠失タンパク質発現細胞を用いて欠失型ウイルスの回収を試みた結果

を示す写真である。F欠損SeVの再構築時に用いたワクシニアウイルスによりヘルパー細胞株からのF蛋白発現が素早くシャットオフしたことが判明した。

1. LLC-MK2およびCV-1はそれぞれの細胞株のみの細胞ライセートを指す。
2. LLC-MK2/F+adおよびCV-1/F+adはアデノウイルスAxCANCreを加えたそれぞれの誘導発現細胞ライセートを指す。
3. LLC-MK2/F-adおよびCV-1/F-adはアデノウイルスAxCANCreを加えていないそれぞれのF遺伝子導入株の細胞ライセートを指す。
4. LLC-MK2/F+ad 3rdはアデノウイルスAxCANCreで誘導発現した細胞をさらに3回継代した細胞のライセートを指す。
5. 1dおよび3dはそれぞれ誘導発現後1日および3日を指す。
6. Vac1dおよびVac 3 dはそれぞれワクシニアウイルス感染後1日および3日の細胞を指す。
7. AraC1dおよびAraC3dはそれぞれAraCを添加して1日および3日の細胞を指す。
8. CHX 1dおよびCHX 3dはそれぞれ蛋白合成阻害剤サイクロヘキシミドを添加して1日および3日の細胞を指す。

図6は、GFP導入F欠失SeV cDNA(pSeV18<sup>+</sup> / $\Delta$ F-GFP)をF非発現LLC-MK2細胞にトランスフェクションしてGFPの発現(RNPの検出)を観察した結果を示す写真である。対照群としてF遺伝子をNP遺伝子の3'末端にシャフルし、F欠失部位にGFPを導入したSeV cDNA(Fシャフル型SeV)を用いた。「all」はSeV cDNAの他に、NP,P,L遺伝子を発現するプラスミド(pGEM/NP, pGEM/P, 及びpGEM/L)も同時にトランスフェクションしたものを表わす。「cDNA」はcDNA (pSeV18<sup>+</sup> / $\Delta$ F-GFP)のみのトランスフェクションを表わす。RNPトランスフェクションはGFPを発現しているPO細胞を回収し、OptiMEM (GIBCO BRL)に懸濁し(10<sup>7</sup> 細胞/ml)、凍結融解3回くり返したライセート100 $\mu$ lをカチオン性リボソームDOSPER(ベーリンガーマインハイム) 25 $\mu$ lと混合し、室温に15分間放置してから、F発現誘導細胞(+ad)に添加し

、RNPトランスフェクションを行った。細胞の対照群としてCre DNAリコンビナーゼを発現する組換えアデノウイルス非添加 (-ad)細胞を用いた。その結果、P0のLLC-MK2細胞ではGFPはSeVウイルスRNPの形成に依存的に発現することが判明し、P1では、F欠失ウイルスはF誘導発現に依存的に増幅されることが判明した。

図7は、F欠失ゲノムcDNAで再構築された機能的なRNPが、F発現ヘルパー細胞でレスキューされ、感染性を有する欠失型ウイルスビリオンを形成し得るかを調べた結果を示す写真である。RNP/oはRNPを重層 (overlay)した細胞を指し、RNP/tはRNPをtransfectionした細胞を指す。

図8は、F欠失ウイルスが、F発現細胞に特異的に増幅されることを確かめた結果を示す写真である。遺伝子欠失型ゲノムから構築した機能的RNPを含むライセートを実施例2に記載のF発現細胞にリポフェクションし、培養上清を回収した。この培養上清をF発現細胞の培地に加え感染させ、3日目に回収された培養上清を、F発現細胞とF非発現細胞に同時に添加し、トリプシン存在と非存在下で3日間培養した。その結果を示す。F発現細胞では、トリプシン存在下でのみウイルスが増幅された。

図9は、F発現細胞に導入した場合に特異的にF欠失ウイルスが培養上清に放出されることを確かめた結果を示す写真である。遺伝子欠失型ゲノムから構築した機能的RNPを含むライセートを実施例2に記載のF発現細胞にリポフェクションし、培養上清を回収した。この培養上清をF発現細胞の培地に加え感染させ、3日目に回収された培養上清を、F発現細胞とF非発現細胞に同時に添加し、トリプシン存在と非存在下で3日間培養した。下段はF非発現細胞の上清の場合の結果を示す。

図10は、F欠失cDNAから回収されたビリオンのゲノム構造を確認するため、F発現細胞の培養上清中のウイルスを回収し、total RNAを抽出して、FとHNをプローブにしてノーザンブロット解析を行った結果を示す写真である。F発現細胞から回収されたウイルスはHN遺伝子は検出されたがF遺伝子は検出されず、F遺伝子

がウイルスゲノム上に存在しないことが明らかとなった。

図 1 1 は、GFP の遺伝子は cDNA の構築の際と同様の F の欠失部位に存在することを示す RT-PCR の結果を示す写真である。1 : +18-NP、+18 Not I サイトの存在の確認。2 : M-GFP、GFP 遺伝子が F 遺伝子欠損部位に存在することの確認。3 : F 遺伝子、F 遺伝子の存在の確認。野生型 SeV と F 欠損 GFP 発現 SeV のゲノム構造を上にした。GFP 遺伝子が F 欠損部位に存在し、NP の 3' 末端に +18 由来の NotI サイトがあり、F 遺伝子が RNA ゲノムのどこにも存在しないことが確認された。

図 1 2 は、ウイルスの F と HN に特異的に反応する金コロイド結合 IgG (antiF, antiHN) を用いた免疫電顕により調べた結果を示す写真である。ウイルスのエンベロープのスパイク様構造は F と HN の蛋白質からなることが明らかとなった。

図 1 3 は、GFP の遺伝子以外の他の遺伝子の構造は野生型と同様であることを確認した RT-PCR の結果を示す図である。

図 1 4 は、F 欠失ウイルス粒子を電顕により、その形態を調べた結果を示す写真である。F 欠失ウイルス粒子は野生型ウイルスと同様に内部にヘリカルな RNP 構造とスパイク様構造を有していた。

図 1 5 は、F 欠失型 SeV ベクターによる *in vitro* での多様な細胞への高効率遺伝子導入の結果を示す写真である。

図 1 6 は、マウス初代骨髓細胞 (BM c-kit+/-) への F 欠失型 SeV ベクターの導入を解析した結果を示す図である。白抜きバーは PE 陽性/GFP 陰性を指し、黒いバーは PE 陽性/GFP 陽性を指す。

図 1 7 は、ラット脳室へのベクターの *in vivo* 投与の結果を示す写真である。

図 1 8 は、F 発現細胞から回収した F 欠損 SeV ウイルスを含む培養上清を F 非発現 LLC-MK2 細胞に感染し、トリブシン存在下または非存在下で 3 日間培養し上清中のウイルスの存在を HA assay で確認した結果を示す写真である。

図 1 9 は、図 1 8 B において発育鶏卵で HA 陽性であった漿尿液 (lane 11 および

lane 12) を発育鶏卵に再接種して培養2日後の漿尿液のHA assayを行った結果を示す写真である。

図20は、HA陽性で感染性がないウイルス液を免疫電顕で調べた結果を示す写真である。ウイルス粒子が確認され、ビリオンのエンベロープは金コロイド標識したHN蛋白を認識する抗体では反応したが、金コロイド標識したF蛋白を認識する抗体では反応しなかった。

図21は、F欠損ウイルス粒子の細胞へのトランスフェクションの結果を示す写真である。

図22は、F、HN共発現細胞の造成をウェスタンブロットにより調べた結果を示す写真である。LLC/VacT7/pGEM/FHNはLLC-MK2細胞にワクシニア感染後、pGEM/FHNプラスミドをトランスフェクションした細胞。LLC/VacT7はワクシニア感染したLLC-MK2細胞。LLCMK2/FHNmixはF、HN遺伝子導入されたLLC-MK2細胞でクロニングしていない細胞。LLC/FHNはLLC-MK2細胞にF、HN遺伝子を導入してアデノウイルスで発現誘導後(3日後)の細胞、1-13、2-6、2-16、3-3、3-18、3-22、4-3、5-9はクロニングしたときの細胞株の番号(名前)を指す。

図23は、pGEM/FHNの添加の有無の違いによるウイルスの形成を確認した結果を示す写真である。FHN欠損GFP発現SeV cDNA, pGEM/NP, pGEM/P, pGEM/L, pGEM/FHNをそれぞれ混合しLLC-MK2細胞に遺伝子導入した。遺伝子導入3時間後培地をAraC, トリプシン入りのMEMに交換し、さらに3日間培養した。遺伝子導入後2日目で蛍光実体顕微鏡で観察し、pGEM/FHNの添加の有無の違いを検証し、GFP発現細胞の広がり度でウイルスの形成を確認した。その結果を示す。再構築時にpGEM/FHNを添加した場合はGFP発現細胞の広がりが確認され、pGEM/FHNの添加がない場合はGFP発現はシングル細胞でしか観察されなかった

図24は、RNPトランスフェクションによるF、HN欠損ウイルスの再構築と増幅を示す写真である。発現誘導後3日目のF HN共発現細胞(12well)にP0 RNPを重層またはDOSPERを用いてリポフェクションし、4日後にGFPを観察した。RNPトラ



ンスフェクションの場合はF欠損と同様にP1のFHN発現細胞でウイルスの回収に成功した(上)。Ade/Creを感染して6時間以後にFHN蛋白が誘導発現された細胞にFHN欠損ウイルス液を感染し増幅ができたことを確認した(下)。

図25は、FHN欠損GFPを発現するcDNAから再構築されたウイルス液はLLC-MK2, LLC-MK2/F, LLC-MK2/HN, LLC-MK2/FHNに感染してトリプシンの添加の有無で培養した結果を示す写真である。培養3日後にGFP蛋白発現細胞の広がりを確認した。その結果を示す。LLC-MK2/FHNでのみGFPの広がりが観察され、このウイルス液はFHN共発現に特異的かつトリプシン依存的に増幅されることが確認された。

図26は、FHN発現細胞の培養上清由来RNAのゲノム構造を確認した結果を示す写真である。

図27は、FHN欠損ウイルスで感染したF発現細胞の培養上清由来RNAのゲノム構造の確認の結果を示す写真である。

図28は、ソラレン・UV照射におけるソラレンの濃度を変化させたときの、ワクシニアウイルスの不活性化とT7活性を示す図である。

図29は、ソラレン・UV照射におけるUV照射時間を変化させたときの、ワクシニアウイルスの不活性化とT7 RNAポリメラーゼ活性を示す図である。

図30は、ソラレン・UV照射したワクシニアウイルスの細胞傷害性(CPE)を示す写真である。 $3 \times 10^5$ のLLC-MK2細胞を6ウェルプレートに播いた。細胞を一晩培養後、ワクシニアウイルスをmoi=2で感染させた。24時間後、CPEを測定した。偽処理のワクシニアウイルスによるCPEはA、15、20、および30分間処理したワクシニアウイルスによるCPEは、それぞれB、C、およびDに示した。

図31は、ワクシニアウイルスのUV処理時間のセンダイウイルス再構成効率に対する影響を示した図である。

図32は、センダイウイルス再構成実験に用いた細胞に残存する複製可能なワクシニアウイルスの力価を示す図である。

図33は、抗VSV-G抗体によるウェスタンブロット解析の結果を示す写真である

。

図34は、抗VSV-G抗体を用いたフローサイトメトリー解析の結果を示す図である。AxCANCre感染4日目のLLC-MK2 VSV-G誘導発現株(L1) (moi=0, 2.5, 5) の解析結果を示す。一次抗体は抗VSV-G抗体 (MoAb I-1)、二次抗体はFITC化 抗マウスIgを用いた。

図35は、AxCANCre の感染量 (MOI=0、1.25、2.5、5、10) を変え、一定量のF 遺伝子を欠失したゲノムを有するシュードタイプセンダイウイルスを感染後上清を回収し、さらにVSV-G 誘導前(-)、誘導後(+)の細胞に感染させ、5日目のGFP の発現している細胞を観察した結果を示す写真である。

図36は、経時的にウイルス産生量を調べた結果を示す写真である。

図37は、VSV-G 発現株を用いて得られたF 遺伝子を欠失したゲノムを有するシュードタイプセンダイウイルスおよびFHN欠損センダイウイルスを抗VSV抗体で処理し、感染性が影響されるを調べた結果を示す写真である。

図38は、GFP遺伝子を含むF、HN 欠失型センダイウイルスを VSV-G遺伝子発現細胞LLCG-L1に感染させ、VSV-Gを外被に有するシュードタイプウイルスの産生が見られるかを GFP遺伝子の発現を指標に調べた結果を示す写真である。

図39は、VSV-G 遺伝子発現細胞で増殖したウイルスが Fおよび HN 欠失型であることを、感染細胞抽出液のタンパク質のウェスタン解析により調べた結果を示す写真である。

図40は、蛍光顕微鏡下でGFP発現細胞を観察した結果を示す写真である。

図41は、エンベロープ発現プラスミドと細胞重層の組み合わせによるSeV/ΔF-GFPの再構成効率の向上を示す図である。P0 (継代前) のd3~d4 (3日目~4日目) において、著しい改善が認められた。

図42は、エンベロープ発現プラスミドと細胞重層の組み合わせによるSeV/ΔF-GFPの再構成の処理条件の検討結果を示す図である。GFP陽性細胞は再構成されたウイルス量を表す。

図4 3は、cDNAからのF欠損センダイウイルスのレスキューの検討結果を示す図である。エンベロープ発現プラスミドと細胞重層の組み合わせによるSeV/ $\Delta$ F-GFPの再構成効率の向上を示す。7日目は全チャレンジとも陽性となるが、成功確率の中程度領域である3日目に着目し、効率の検討を行った。

図4 4は、GFPを含まないLacZ搭載 F 欠失型センダイウイルスベクターのlacZの発現を示す写真である。

図4 5は、センダイウイルスゲノムcDNA断片のサブクローニング (A) と新たにNotIサイトを導入し構築した5種類のセンダイウイルスゲノムcDNAの構造 (B) を示す図である。

図4 6は、SEAPにNotIサイト、転写開始シグナル、介在配列、転写終結シグナルを付加するためのクローニング用プラスミドの構造を示す図である。

図4 7は、各センダイウイルスベクターのブランクアッセイの結果を示す写真である。LAS1000で取り込んだブランクアッセイの蛍光画像の一部を示す。

図4 8は、各センダイウイルスベクター間におけるレポーター遺伝子 (SEAP) の発現量の違いを比較した結果を示す図である。SeV18+/SEAPのデータを100としてそれぞれ相対値を表した。SEAP遺伝子が下流に位置するに従ってその活性すなわち発現量が低下していくことがわかった。

図4 9は、P1 FHN共発現細胞におけるG F P 発現を示す顕微鏡写真である。

図5 0は、VSV-GシュードタイプSeV/ $\Delta$ F:GFP感染細胞の抽出液を、抗F抗体 (anti-F)、抗HN抗体 (anti-HN)、抗センダイウイルス抗体 (anti-SeV) を用いてウェスタンブロット解析を行った結果を示す写真である。

図5 1は、中和抗体 (VGV抗体) の存在下または非存在下でFおよびHNを欠損したVSV-GシュードタイプSeVを感染させた細胞のGFPの蛍光を示す写真である。

図5 2は、密度勾配超遠心法を用いて分画したF遺伝子あるいはF, HN遺伝子を欠失したゲノムを有するVSV-Gシュードタイプセンダイウイルスのウェスタン解析の結果を示す写真である。

図5 3は、F遺伝子を欠損したゲノムを有するセンダイウイルス、あるいはF遺伝子またはF,HN遺伝子を欠失したゲノムを有するVSV-Gシュードタイプセンダイウイルスによる赤血球凝集反応を示す写真である。

図5 4は、F遺伝子を欠失したゲノムを有するセンダイウイルスまたはVSV-Gシュードタイプセンダイウイルスによる培養細胞への感染特異性を示す図である。

図5 5は、NGF発現を搭載したF欠失型センダイウイルス (NGF/SeV/ $\Delta$ F) の構造の確認を示す写真である。

図5 6は、NGF搭載F欠失型SeV感染細胞より発現されるNGFの活性を示す図である。ニワトリの後根神経節の初代神経細胞分散培養系に、培養開始と同時にSeV感染細胞の培養上清希釈液或いはコントロールとしてのNGF蛋白を添加し、3日後にミトコンドリアによる還元活性を指標として生細胞を定量した (n=3)。培養上清は1/1000希釈相当量添加した。

図5 7は、NGF搭載F欠失型SeV感染細胞より発現されるNGFの活性を示す写真である。ニワトリの後根神経節の初代神経細胞分散培養系に、培養開始と同時にSeV感染細胞の培養上清希釈液或いはコントロールとしてのNGF蛋白を添加し、3日後に検鏡した。

- A) コントロール (NGF添加無し)、
- B) NGF蛋白10ng/mL添加、
- C) NGF/SeV感染細胞培養上清1/100希釈添加、
- D) NGF/SeV感染細胞培養上清1/100希釈添加、
- E) NGF/SeV/ $\Delta$ F感染細胞培養上清1/100希釈添加、
- F) NGF/SeV/ $\Delta$ F-GFP感染細胞培養上清1/100希釈添加

図5 8は、Ad-CreのmoiとF蛋白の発現量を示す写真である。

図5 9は、Adeno-CreによるLLC-MK2/Fの発現を示す写真である。

図6 0は、継代による発現の持続性を示す写真である。

図6 1は、継代によるF蛋白の局在化を示す写真である。

図 6 2 は、GFP-CIU と抗SeV-CIU との相関関係を示す図である。

#### 発明を実施するための最良の形態

以下実施例により本発明を具体的に説明するが、本発明はこれらの実施例に限定されるものではない。

##### [実施例 1] F 欠失型センダイウイルスの構築

###### <1> F 欠失型SeVゲノムcDNAおよびF発現プラスミドの構築

センダイウイルス (SeV) 全長ゲノムcDNA、pSeV18<sup>+</sup> b(+) (Hasan, M. K. et al., 1997, J. General Virology 78: 2813-2820) (「pSeV18<sup>+</sup> b(+)」は「pSeV18<sup>+</sup>」ともいう) のcDNAをSphI/KpnIで消化してフラグメント(14673bp)を回収し、pUC18にクローニングしてともいうプラスミドpUC18/KSとした。F欠損部位の構築はこのpUC18/KS上で行った。F遺伝子の欠損は、PCR-ライゲーション方法の組み合わせで行い、結果としてF遺伝子のORF (ATG-TGA=1698bp) を除いてatgcatgccggcagatga (配列番号: 1) で連結し、F 欠失型SeVゲノムcDNA (pSeV18<sup>+</sup>/ΔF) を構築した。PCRは、Fの上流には (forward: 5'-gttgagtactgcaagagc/配列番号: 2, reverse: 5'-tttccggcatgcatgtttcccaaggggagagttttgcaacc/配列番号: 3)、F遺伝子の下流には (forward: 5'-atgcatgccggcagatga/配列番号: 4, reverse: 5'-tgggtgaatgagagaatcagc/配列番号: 5) のPCR産物をEcoT22Iで連結した。このように得られたプラスミドをSacIとSalIで消化して、F欠損部位を含む領域の断片 (4931bp) を回収してpUC18にクローニングし、pUC18/dFSSとした。このpUC18/dFSSをDraIIIで消化して、断片を回収してpSeV18<sup>+</sup>のF遺伝子を含む領域のDraIII断片と置き換え、ライゲーションしてプラスミドpSeV18<sup>+</sup> /ΔF を得た。

さらに、F欠失部位にEGFP遺伝子を搭載したcDNA (pSeV18<sup>+</sup>/ΔF-GFP) を構築するため、PCRにより、EGFP遺伝子の増幅を行った。EGFP遺伝子を6の倍数(Hausmann, S. et al., RNA 2, 1033-1045 (1996)) に合わせるため 5'はNsiI-taildプライマー (5'-atgcatatggtgatgcggttttggcagtag: 配列番号: 6)、3'はNgoMIV-tailedプ

ライマー(5'-Tgccggctattattacttgtacagctcgc: 配列番号: 7)を用いてPCRを行った。PCR産物を制限酵素NsiI とNgoMIVで消化してゲルから断片を回収し、pUC18/dFSSのF欠失部位にあるNsiI とNgoMIVという制限酵素部位に連結し、シーケンスを確認した。ここから、EGFP遺伝子を含むDraIII断片を回収し、pSeV18<sup>+</sup>のF遺伝子を含む領域のDraIII断片と置き換え、ライゲーションしてプラスミドpSeV18<sup>+</sup>/ΔF-GFPを得た。

一方、F遺伝子を発現するCre/loxP誘導型発現プラスミドの構築はSeV F遺伝子をPCRで増幅し、シーケンスを確認した後、Cre DNAリコンビナーゼにより遺伝子産物を誘導発現されるように設計されたプラスミドpCALNdLw(Araiら J. Virology72,1998,p1115-1121)のユニークサイト SwaI部位に挿入し、プラスミドpCALNdLw/Fとした。

#### <2> SeV-F蛋白を誘導発現するヘルパー細胞の作製

F欠損ゲノムから感染ウイルス粒子を回収するため、SeV-F蛋白を発現するヘルパー細胞株を樹立した。細胞はSeVの増殖によく用いられているモンキー腎臓由来細胞株、LLC-MK2細胞を用いた。LLC-MK2細胞は、10%の熱処理した不活化ウシ胎児血清(FBS)、ペニシリンGナトリウム 50単位/ml、およびストレプトマイシン 50 μg/mlを添加したMEMで37°C、5% CO<sub>2</sub>で培養した。SeV-F遺伝子産物は細胞傷害性を有するため、Cre DNAリコンビナーゼによりF遺伝子産物を誘導発現されるように設計された上記プラスミドpCALNdLw/Fを、リン酸カルシウム法 (mammalian transfection kit (Stratagene)) により、そのプロトコールに従ってLLC-MK2細胞に遺伝子導入を行った。

10cmプレートを用い、40%コンフルエントまで生育したLLC-MK2細胞に10 μgのプラスミドpCALNdLw/Fを導入後、10mlの10% FBSを含むMEM培地にて、37°Cの5% CO<sub>2</sub> インキュベーター中で24時間培養した。24時間後に細胞をはがし、10ml培地に懸濁後、10cmシャーレ5枚を用い、5ml 1枚、2ml 2枚、0.2ml 2枚に蒔き、G418 (GIBCO-BRL)を1200 μg/mlを含む10mlの10%FBSを含むMEM培地にて培養を行い、2

日毎に培地交換しながら、14日間培養し、遺伝子の安定導入株の選択を行った。該培地により生育してきたG418に耐性を示す細胞はクローニングリングを用いて30株を回収した。各クローンは10cmプレートでコンフルエントになるまで拡大培養を続けた。

各クローンについてCre DNAリコンビナーゼを発現する組み換えアデノウイルスAxCANCreで感染後、抗SeV-F蛋白質モノクローナルIgG (f236, J. Biochem. 123: 1064-1072) を用いてSeV-F蛋白の発現をウエスタンブロット法により以下のように調べた。

各クローンは6cmシャーレにてコンフルエントまで生育させた後、アデノウイルスAxCANCreを斉藤らの方法 (Saito et al., Nucl. Acids Res. 23: 3816-3821 (1995); Arai, T. et al., J Virol 72, 1115-1121 (1998)) により moi=3 で感染後、3日間培養した。該細胞は培養上清を取り除いた後、PBS緩衝液で2回洗浄し、スクレーパーで細胞をはがし、1500×gで5分間遠心し、細胞を集めた。

該細胞は-80℃で保存し、必要に応じて解凍して使用することができる。集めた細胞は150μl PBSバッファーに懸濁後、同量の2×Tris-SDS-BME sample loading buffer (0.625M Tris, pH 6.8, 5%SDS, 25% 2-ME, 50% glycerol, 0.025%BPB, 0.1%社製) を加え、98℃ 3分間加熱処理後電気泳動用試料に供した。該試料 (1レーン当たり $1 \times 10^5$  細胞) をSDS-ポリアクリルアミドゲル (マルチゲル10/20、第一化学社製) を用い、電気泳動により分画し、分画された蛋白はセミドライブロット法によりPVDF転写膜 (Immobilon-P transfer membranes, Millipore社製) に転写した。転写は100% メタノールに30秒、水に30分間浸した転写膜を使用し、 $1\text{mA}/\text{cm}^2$  定電流の条件で1時間行った。

該転写膜を0.05%Tween20, 1%BSAを添加したブロッキング溶液 (ブロックエース、雪印社製) 中で1時間振蕩後、0.05%Tween20, 1%BSAを添加したブロッキング溶液で1/1000希釈した抗SeV-F抗体 (f236) で室温で2時間反応させた。該転写膜を3回20mlのPBS-0.1%Tween20に5分間振蕩して洗浄した後、PBS緩衝液で5分

間振蕩し洗浄した。該転写膜を0.05%Tween20, 1%BSAを添加したブロッキング溶液で1/2000希釈したパーオキシダーゼで標識した抗マウスIgG抗体 (Goat anti-mouse IgG, Zymed社製) 10mlを室温で1時間反応させた。該転写膜を3度20mlのPBS-0.1%Tween20に5分間振蕩して洗浄した後、PBS緩衝液で5分間振蕩し洗浄した。

化学発光法 (ECL western blotting detection reagents, Amersham社製) により抗SeV-F抗体と交叉のみられる該転写膜上の蛋白質の検出を行った。結果は図1に示す。AxCANCre感染特異的なSeV-Fの発現が検出され、SeV-F遺伝子産物を誘導発現するLLC-MK2細胞の作出が確認された。

得られた数細胞株の内の一つのLLC-MK2/F7細胞を抗SeV-F抗体を用いてフローサイトメトリー解析を行った (図2)。すなわち、 $1 \times 10^5$ 細胞を15,000rpm 4℃で5分間スピンドウンし、PBS 200 $\mu$ lで洗浄し、100倍希釈した抗Fモノクローナル抗体 (f236)、0.05%アジ化ナトリウム、2%FCSを含むFACS用PBS (日研化学) で4℃、1時間遮光して反応させた。再び15,000rpm 4℃で5分間スピンドウンし、PBS 200 $\mu$ lで洗浄し、FITC標識した抗マウスIgG (CAPPEL社) 1 $\mu$ g/mlと30分間氷上で反応させ、再びPBS 200 $\mu$ lで洗浄し、15,000rpm 4℃で5分間遠心して細胞をスピンドウンし、1mlのFACS用PBSに懸濁した。EPICS ELITE (コールター社製) アルゴンレーザーを用いて、励起波長488nm、蛍光波長525nmで解析した。その結果、LLC-MK2/F7 では SeV-F遺伝子誘導発現時特異的に抗体との高い反応性が検出され、SeV-F蛋白質が細胞表面に発現されることが確認された。

#### 【実施例2】 ヘルパー細胞で発現されたSeV-Fタンパク質の機能確認

ヘルパー細胞で誘導発現されたSeV-F蛋白質は従来の蛋白機能が保たれているかを調べた。

LLC-MK2/F7細胞を6cmシャーレに蒔き、コンフルエントまで生育させた後、アデノウイルスAxCANCreを斉藤らの方法 (上記) によりmoi=3で感染後、トリプシン (7.5 $\mu$ g/ml, GIBCOBRL)を含むMEM (serum free)で37℃ 5%CO<sub>2</sub> インキュベーター



で3日間培養した。

該細胞は培養上清を取り除いた後、PBS緩衝液で2回洗浄し、スクレーパーで細胞をはがし、1500×gで5分間遠心し、細胞を集めた。前述したウエスタンブロット法により発現されたF蛋白質のトリプシンによる解裂を確認した(図3)。SeV-F蛋白質は非活性型の前駆蛋白のF0として合成され、トリプシンの蛋白分解作用により、F1とF2の2つのサブユニットに解裂し活性化される。このようにF蛋白が誘導発現後のLLC-MK2/F7細胞は普通の細胞同様に継代してもF蛋白が持続的に発現し、発現されたF蛋白による細胞傷害性が観察されず、F蛋白発現細胞同士での細胞融合も観察されなかった。しかし、このF発現細胞にSeV-HN発現プラスミド(pCAG/SeV-HN)をトランスフェクションしてトリプシンを含むMEMで3日間培養すると、細胞間の融合が多く観察された。細胞表面におけるHNの発現は赤血球の細胞表面への吸着実験(Hematoadsorption assay; Had assay)で確認した(図4)。すなわち、培養細胞に1%ニワトリ赤血球を1ml/dishを加え、4℃で10分間静置した後、細胞をPBS緩衝液で3回洗浄したところ、細胞表面の赤血球のコロニーが観察された。赤血球凝集した細胞で細胞融合が観察され、F蛋白はHNと相互作用して細胞融合を引き起こしたことが判明し、LLC-MK2/F7で持続発現しているF蛋白は従来の機能を保っていることが示された。

### [実施例3] F欠失型ゲノムを持つ機能的RNPおよびビリオンの形成

欠失型ウイルスの回収ではビリオンの回収は欠失タンパク質発現細胞を使う必要がある。ところが、欠失タンパク質発現細胞を用いて欠失型ウイルスの回収を試みたところ、F欠損SeVの再構築時に用いたワクシニアウイルスによりヘルパー細胞株からのF蛋白発現が素早くシャットオフしたことが判明し(図5)、ヘルパー細胞株から直接のF蛋白の供給によるウイルスの再構成に成功しなかった。ワクシニアウイルスに対するソラレン(psoralen)添加で長波長紫外線(long-waveUV)での処理(PLWUV処理)は、ワクシニアウイルスの複製能力を失活させ、T7発現活性が損なわれないことが報告されている(Tsungら、J Virol 70,165-171、1996

)。そこで、このPLWUV処理したワクシニアウイルス (PLWUV-VacT7) を用いてウイルスの再構築を試みた。紫外線照射装置は、15ワットバルブを5本が装備された UV Stratakinker 2400 (カタログ番号 400676 (100V), ストラタジーン社, La Jolla, CA, USA) を用いた。その結果、再構築に用いた F 発現細胞から F 蛋白の発現は阻害されたものの、このPLWUV-VacT7で再構築した細胞のlysateをヘルパー細胞へ感染してもaraCの存在下ではワクシニアが殆ど増殖せず、ヘルパー細胞株からのF蛋白発現にも殆ど影響しないことが判明した。さらに、このPLWUV-VacT7を用いた組み換え野生型SeVの再構築では従来が $10^5$ 以上の細胞がないとウイルスが回収されなかったのに対し、 $10^3$ の細胞からもウイルス回収が可能となり、ウイルスの再構築の効率が大きく改善された。この方法を用いて、F欠失SeVウイルスの再構築を試みた。

#### <F 欠失SeVウイルスの再構築及び増幅>

F欠損部位にenhanced green fluorescent protein(EGFP) 遺伝子をレポーターとして6nルールに従って導入した上記 pSeV18'/ $\Delta$ F-GFP を下記のようにして LLC-MK2細胞にトランスフェクションしてGFPの発現を観察した。この時RNP形成に必要な構成要素である、3つのウイルス由来遺伝子NP、P、Lの有無による影響も検討した。

LLC-MK2 細胞を $5 \times 10^6$ cells/dish で 100mm ペトリ皿に蒔き、24時間培養後、ソラレンと長波長紫外線 (365nm) で 20 分間処理し、T7 RNAポリメラーゼを発現するリコンビナントワクシニアウイルス (Fuerst, T.R. et al., Proc. Natl. Acad. Sci. USA 83, 8122-8126 (1986)) に室温で1時間感染させた (moi=2) (moi=2~3、好適にはmoi=2が用いられる)。細胞を3回洗浄してからプラスミド pSeV18'/ $\Delta$ F-GFP, pGEM/NP, pGEM/P, 及びpGEM/L(Kato, A. et al., Genes cells 1, 569-579 (1996)) をそれぞれ12 $\mu$ g, 4 $\mu$ g, 2 $\mu$ g, 及び4 $\mu$ g /dish の量比で OptiMEM(GIBCO)に懸濁し、SuperFect transfection reagent(1 $\mu$ g DNA/5 $\mu$ l の SuperFect, QIAGEN)を入れて混合し、室温で10 分間放置後、最終的に3%FBSを含

OptiMEM 3mlに入れ、細胞に添加して培養した。pSeV18'/ $\Delta$ F-GFP の代わりに対照として野生型SeVゲノムcDNA (pSeV(+)) (Kato, A. et al., Genes cells 1, 569-579 (1996)) を用いて同様の実験を行った。3時間培養後、細胞を、血清を含まないMEM で2回洗浄し、シトシン $\beta$ -D-アラビノフラノシド40 $\mu$ g/ml (AraC, Sigma), トリプシン7.5 $\mu$ g/ml (GIBCO) を含むMEMで70時間培養した。これらの細胞を回収し、ペレットをOptiMEM に懸濁した ( $10^7$  cells/ ml )。凍結融解を3回繰り返してlipofection reagent DOSPER (Boehringer mannheim)と混合し ( $10^6$ cells/25 $\mu$ l DOSPER) 室温で15分放置した後、F発現LLC-MK2/F7 細胞株にトランスフェクション ( $10^6$ cells /well 12-well-plate) し、血清を含まないMEM (40 $\mu$ g/ml AraC, 7.5 $\mu$ g/ml トリプシンを含む)で培養した。

その結果、ウイルス由来の3つの構成要素、NP、P、Lがすべて揃ったときにのみGFPの発現が認められ、外来遺伝子を発現する欠失ウイルスRNPが形成し得ることが判明した (図6)。

#### <F欠失型ビリオンの確認>

上記のようにしてF欠失ゲノムcDNAで再構築された機能的なRNPが、F発現ヘルパー細胞でレスキューされ、感染性を有する欠失型ウイルスビリオンを形成し得るかを調べた。前述したように機能的RNPを形成される条件 (pSeV18'/ $\Delta$ F-GFP, pGEM/NP, pGEM/P, 及びpGEM/Lを同時にトランスフェクトする条件) と形成されない条件 (pSeV18'/ $\Delta$ F-GFP, pGEM/NPの2種のプラスミドのみをトランスフェクトする条件) で再構築を行った細胞を凍結/融解して得たライセートをカチオニックリボソームと混合しF発現細胞と非発現細胞にそれぞれリポフェクションし、これらの細胞におけるGFP発現細胞の広がりやウイルス粒子の形成を観察した。その結果、機能的RNPが再構築された条件のライセートを用い、F発現細胞に導入した際にのみGFP発現細胞の広がりが観察された (図7)。さらに、ブランクアッセイにおいても、同様の条件でのみブランクの形成が観察された。これらの結果から、F欠損ウイルスゲノムから形成された機能的RNPがF発現細胞由来のFタンパク質

の存在下で、さらに感染性ウイルス粒子として形成され、細胞外に放出されたことが明らかになった。

培養上清中の感染性F欠失型ビリオンの存在は以下の実験により証明された。F遺伝子欠失型ゲノムから構築した機能的RNPを含むライセートを実施例2に記載のF発現細胞にリポフェクションし、培養上清を回収した。この培養上清をF発現細胞の培地に加え感染させ、3日目に回収された培養上清を、F発現細胞とF非発現細胞に同時に添加し、トリプシン存在と非存在下で3日間培養した。F発現細胞では、トリプシン存在下でのみウイルスが増幅された(図8)。F非発現細胞の上清(図9 下段)、またはトリプシン非存在下で培養したF発現細胞からは感染性を持たないウイルス粒子が放出されていることが明らかとなった。以上のことをまとめると、F欠損GFP発現ウイルスはF発現細胞に特異的かつトリプシン解裂に依存的に増幅されることが明らかとなった。このように増幅された感染性F欠失型セグメンタイルウイルスのタイターは $0.5 \times 10^7 \sim 1 \times 10^7$  CIU/mlの範囲にあった。

#### 【実施例4】 F欠失型GFP発現ウイルスの解析

F欠失cDNAから回収されたビリオンのゲノム構造を確認するため、F発現細胞の培養上清中のウイルスを回収し、total RNAを抽出して、FとHNをプローブにしてノーザンブロット解析を行った。その結果F発現細胞から回収されたウイルスはHN遺伝子は検出されたがF遺伝子は検出されず、F遺伝子がウイルスゲノム上に存在しないことが明らかとなった(図10)。さらにRT-PCRによりGFPの遺伝子はcDNAの構築の際と同様のFの欠失部位に存在すること(図11)、また、他の遺伝子の構造は野生型と同様であることを確認した。以上のことからウイルス再構成中にゲノムの再編成は起きていないことが示された。また、回収されたF欠失ウイルス粒子を電顕により、その形態を調べた。F欠失ウイルス粒子は野生型ウイルスと同様に内部にヘリカルなRNP構造とスパイク様構造を有していた(図14)。さらに、ウイルスのFとHNに特異的に反応する金コロイド結合IgG(antiF, antiHN)を用いた免疫電顕により調べたところ、ウイルスのエンベロープのスパイク様構造

はFとHNの蛋白質からなることが明らかとなり(図12)、ヘルパー細胞の生産するF蛋白質がこのビリオンに効率的に取り込まれていることがわかった。以下に詳述する。

<Total RNAの抽出、ノーザンブロット解析、およびRT-PCR>

F発現細胞LLC-MK2/F7にウイルス感染して3日目の培養上清からQIAamp Viral RNA mini kit(QIAGEN)を用い、そのプロトコールに従いtotal RNAの抽出を行った。精製したtotal RNA (5 $\mu$ g) をホルムアルデヒドを含む1%変性アガロースゲルにて泳動分離してから、バキュームブロッティング装置 (Amersham Pharmacia 社)を用いHybond-N+メンブランにトランスファした。作成したメンブランは0.05MのNaOHで固定し、2倍希釈したSSC緩衝液 (Nacalai tesque) ですすいだ後、ハイブリダイゼーション溶液 (Boehringer Mannheim) で30分間プレハイブリダイゼーションを行った。ジゴキシゲニン (DIG) -dUTP(アルカリ感受性) を用いたランダムプライムDNA標識法(DIG DNA Labeling Kit, Boehringer mannheim)により作成したFあるいはHN遺伝子のプローブを添加して16時間ハイブリダイズさせた。その後、メンブランを洗浄して、アルカリフォスフォターゼ標識抗DIG抗体 (anti-digoxigenin-AP)と反応させ、DIG detection kitを用いて解析した。その結果F発現細胞から回収されたウイルスはHN遺伝子は検出されたがF遺伝子は検出されず、F遺伝子がウイルスゲノム上に存在しないことが明らかとなった(図10)。

さらにRT-PCRにより詳細な解析を行った。RT-PCRは精製したウイルスRNAをSUPERScriptII Preamplification System(Gibco BRL)を用い、そのプロトコールに従いfirst strand cDNA を合成し、LA PCR kit (TAKARA ver2.1)を用いて次のような条件でPCRを行った。94°C/3分反応後、94°C/45秒,55°C/45秒,72°C/90秒を1サイクルとして30サイクルを増幅して72°Cで10分間置き、2%アガロースゲルで100V/30分電気泳動してエチジウムブロマイド染色し、撮影した。M遺伝子とF欠失部位に挿入したEGFPの確認に用いたプライマーはforward 1 :5'-

atcagagacctgcgacaatgc (配列番号: 8), reverse 1: 5'-aagtcgtgctgcttcattgttg (配列番号: 9)、F 欠失部位に挿入したEGFPとHN遺伝子の確認に用いたプライマーはforward 2: 5'-acaaccactacctgagcaccagtc (配列番号: 10), reverse 2: 5'-gcctaacacatccagagatcg (配列番号: 11)、さらに、M遺伝子とHN遺伝子との間はforward 3: 5'-acattcatgagtcagctcgc (配列番号: 12) とreverse 2プライマー (配列番号: 11) で行った。その結果、GFPの遺伝子はcDNAの構築の際と同様のFの欠失部位に存在すること (図11)、また、他の遺伝子の構造は野生型と同様であることを確認した (図13)。以上のことからウイルス再構成中にゲノムの再編成は起きていないことが示された。

#### <金コロイド免疫標識電顕解析>

回収されたF欠失ウイルス粒子を電顕により、その形態を調べた。まず、欠損型ウイルス感染細胞の培養上清を28,000rpm、30分間遠心してウイルスをペレットにした後で、 $1 \times 10^9$  HAU/mlになるように10倍希釈したPBSに再懸濁し、その一滴を支持膜付きのマイクログリット上に滴下して室温で乾燥させた。3.7%ホルマリンを含むPBSにより15分間固定処理後、0.1%BSAを含むPBS溶液で30分前処理をし、さらに同溶液で200倍希釈した抗Fモノクローナル抗体 (f236)、または抗HNモノクローナル抗体 (Miura, N. et al., Exp. Cell Res. (1982) 141: 409-420) を滴下して保湿状態で60分間反応させた。その後グリットをPBSで洗浄して、200倍希釈した金コロイド標識抗マウスIgG抗体を滴下して同じく保湿状態で60分間反応させた。続いてグリットをPBS、滅菌蒸留水の順で洗浄し室温で風乾後、グリットの上に4%の酢酸ウラニウム溶液で2分間染色し乾燥させた上、JEM-1200EXII 電子顕微鏡 (日本電子) を用いて観察、撮影した。その結果、ウイルスのエンベロープのスパイク様構造はFとHNのタンパク質からなることが明らかとなり (図12)、ヘルパー細胞の生産するFタンパク質がこのビリオンに効率的に取り込まれていることがわかった。また、F欠失ウイルス粒子は野生型ウイルスと同様に内部にヘリカルなRNP構造とスパイク様構造を有していた (図14)。

[実施例 5] F欠失型SeVベクターによるin vitroでの多様な細胞への高効率遺伝子導入

<ラット大脳皮質神経細胞の初代培養細胞への導入>

ラット大脳皮質神経細胞の初代培養細胞を、以下のようにして調製し培養した。妊娠18日SDラット (SPF/VAF Crj: CD, 雌, 332g, ~9 週 Charles River)をジエチルエーテルにより深麻酔し、腋下動脈放血により安楽死させた。開腹し子宮から胎児を摘出し皮膚頭蓋を切り開き脳を取り出した。実体顕微鏡下で大脳半球を作業液 (5%ウマ血清と5%子牛血清、10%DMSOを含む) DMEMに移し、スライスして氷温冷却したパパイン溶液 (1.5U, シスチン0.2mg、ウシ血清アルブミン0.2mg、グルコース5mg、DNase 0.1mg/ml) を加え、32°Cで5分毎に転倒攪拌して15分間インキュベーションした。懸濁液が十分濁り、組織片が半透明になったことを確認して組織片がばらばらになるまでピペティングを繰り返した。32°Cにて1200rpm 5分間遠心した後、細胞をB27 supplement添加したneural basal medium (GibcoBRL, Burlington, Ontario, Canada)に再懸濁し、ポリ-d-リジン (Becton Dickinson Labware, Bedford, MA, U.S.A.) でコーティングされたプレート上に  $1 \times 10^5$  cells/dish 蒔き、37°C、5 %CO<sub>2</sub> で培養を行った。

その大脳皮質初代培養神経細胞  $5 \times 10^5$ /wellを5日間培養後、F欠失型SeVベクターを感染させ (moi=5)、さらに3日間培養した。1% パラホルムアルデヒド、5% ヤギ血清、0.5% Triton-Xを含む固定液で5分間室温で固定し、BlockAce(雪印乳業)にて室温2時間ブロッキングして500倍に希釈されたヤギ抗ラット microtubule-associated protein 2 (MAP-2) (Boehringer) IgGと室温で1時間インキュベーションした。PBS(-)で15分毎に3回洗浄後、5%ヤギ血清/PBSで100倍希釈されたcys3-結合抗マウスIgGと室温で1時間インキュベーションした。さらにPBS(-)で15分毎に3回洗浄後、細胞にVectashield mounting medium (Vector Laboratories, Burlingame, U.S.A.)を加え、共焦点顕微鏡 (Nippon Bio-Rad MRC 1024, Japan)で 470-500-nm または 510-550-nm のexcitation

band-pass filter を付けた Nikon Diaphot 300 倒立顕微鏡でMAP-2の免疫染色と GFPの蛍光による2重染色の蛍光観察を行った。その結果、MAP2 陽性神経細胞にはGFPがほぼ100%導入されたことが明らかとなった (図15)。

#### <正常ヒト細胞への導入>

正常ヒト平滑筋細胞、正常ヒト肝細胞、正常ヒト肺毛細血管内皮細胞 (セルシステムズ) は大日本製薬から購入し、SFM CS-C培地キット (セルシステムズ) で 37°C、5% CO<sub>2</sub>で培養した。

正常ヒト平滑筋細胞 (図15, Muscle)、正常ヒト肝細胞 (図15, Liver)、正常ヒト肺毛細血管内皮細胞 (図15, Lung)等のヒト正常細胞にF欠失型SeVベクターを感染して (m.o.i=5)、GFP発現を観察した。いずれの細胞においてもほぼ100%の導入効率で強力なGFP遺伝子発現をしていることが確認された (図15)。

#### <マウス初代骨髄細胞への導入>

さらに、マウス初代骨髄細胞をリンネジマーカーで分離して、F欠失型SeVベクターを感染させる実験を行った。まず、C57BL マウス (6週令雄) に150mg/kg になるように5-fluorouracil (5-FU, Wako Pure Chemical Industries) を腹腔内注射 (IP injection) し、投与2日後、大腿骨より骨髄細胞を回収した。Lympholyte-M (Cedarlane)を用いた密度勾配遠心によって単核細胞を分離した。3x10<sup>6</sup>の単核細胞に対し、ビオチン標識された抗CD45R (B220), 抗Ly6G (Gr-1), 抗Ly-76 (TER-119), 抗I (Thy1.2), 抗Mac-1を結合させたストレプトアビジン磁気ビーズ (ファーマジェン社, フナコシ社) の混合したものを3x10<sup>7</sup>を加え4°Cにて1時間反応させ、磁石により、Lin<sup>+</sup>の細胞を除いた分画を回収した (Lin<sup>-</sup>細胞) (Erich, S. et al., Blood 1999. 93 (1), 80-86)。Lin<sup>-</sup>細胞4x10<sup>5</sup>細胞に対し、2x10<sup>7</sup> HAU/ml のSeVを加え、さらに、組換えラットSCF (100ng/ml, BRL), 組換えヒトIL-6 (100U/ml)を加えた。また8x10<sup>5</sup>のトータル骨髄細胞に対してF欠損SeV 4x10<sup>7</sup>HAU/ml、1x10<sup>6</sup>の細胞に対し5x10<sup>7</sup>HAU/mlのGFP-SeVを加えた。なお、GFP-SeVは、SeV転写ユニットpUC18/T7HVJRz.DNA(+18)(Genes Cells, 1996, 1:569-579)の制



限酵素NotI開裂部位に、緑色蛍光タンパク質(GFP)遺伝子(構造遺伝子長717bp)に転写開始(R1)と終結(R2)シグナルと介在(IG)配列を付加したNotI断片をPCRにより増幅させ、導入して作製した。既知の方法(Genes Cells, 1996, 1:569-579)に従い、LLC-MK2細胞および発育鶏卵を用いてGFP遺伝子を含むウイルスの再構築を行い、目的の遺伝子を含むウイルスを回収した。GFP-SeVを感染して48時間培養した後、細胞をそれぞれ2群に分け、一つにはフィコエリスリン(phycoerythrin)(PE)標識抗-CD117(c-kit, Pharmingen)を1時間反応させ、もう一群は対照群とした。3回PBSにて洗浄した後、フローサイトメータ(EPICS Elite ESP; Coulter, Miami, FL)による解析を行った。

その結果、血液のprimitive幹細胞のマーカーである抗c-kit抗体でエンリッチした骨髓細胞にもF欠失型SeVベクターは感染し、GFP遺伝子発現が観察された(図16)。培養上清中の感染性粒子の確認は、細胞培養上清をトリプシンで処理後、LLC-MK2細胞に添加し、3日後にGFP発現細胞の存在の有無により行った。これらの細胞のいずれにおいても感染性のあるウイルス粒子が放出されていないことが確認された。

#### 〔実施例6〕 ラット脳室へのベクターの投与

ラット(F334/Du Crj, 6週令、雌、Charles River)に生理食塩水(大塚製薬)で10倍希釈した(5mg/ml)ネンブタールナトリウム溶液(ダイナボット)を腹腔内注射により麻酔し、小動物用脳定位固定装置(DAVID KOPF社)を用いてウイルスの投与を行った。投与部位はinteraural lineよりブレグマ(bregma)へ5.2mm、ラムダより右耳へ2.0mm、脳表面より2.4mmの位置に30Gの交換針(Hamilton社)で20 $\mu$ l (10<sup>8</sup> CIU) 注入した。すると脳室の上皮細胞にGFPの高い発現が観察された(図17)。さらに、F欠失型SeVベクターでは注射部位の周辺のウイルスが接触しうる上皮細胞または神経細胞にしかGFPタンパク質の発現が観察されず、これらの部位に病変所見が観察されなかった。投与されたラットでは解剖されるまでに外見的な行動異常や体重変化などが観察されず、解剖後各臓器、脳のほか、肝臓

、肺、腎臓、心臓、脾臓、胃、腸等の組織器官のいずれにおいても病変所見が観察されなかった。

【実施例 7】 F欠損SeVゲノムからのF-lessウイルス粒子の形成

<1>

F非発現LLC-MK2細胞およびF発現LLC-MK2細胞(LC-MK2/F7)にF欠損SeVウイルスを感染し、トリプシン存在下(+)と非存在下(-)で培養して3日後の細胞培養上清のHA assayの結果を示した(図18A)。これらの培養上清をそれぞれ発育鶏卵に接種し、2日培養後の鶏卵の漿尿液のHA assayの結果を示した(図18B)。パネル上部の「C」は対照群として用いたPBSを表わす。Dilution(希釈)の数字はウイルス液の希釈倍率を表わす。さらに、発育鶏卵でHA陽性であった漿尿液(lane 11および lane 12)を発育鶏卵に再接種して培養2日後の漿尿液のHA assayを行った(図19C)。この結果、F欠損SeVウイルスを感染したF非発現細胞または発育鶏卵ではHAが陽性にもかかわらず、発育鶏卵に再接種してもウイルスが全く増幅されず、このHA陽性のウイルス液が二次感染性がないものと判明した。

<2>

F非発現細胞で増幅された非感染性ウイルス液にウイルス粒子が存在するかについて検討した。F発現細胞の培養上清、HA陽性で非感染性漿尿液、および野生型SeVからQIAamp viral RNA mini kit(QIAGEN)により調製したtotal RNAをF遺伝子とHN遺伝子をプローブとして用いてノーザンブロッティングを行った。その結果、漿尿液、またはF発現細胞の培養上清のウイルス由来のRNAのいずれもHN遺伝子のプローブでバンドが検出されたが、F遺伝子のプローブでバンドが検出されなかった(図10)。このHA陽性で感染性がない液にはF欠損ゲノムを持っている非感染性のウイルス様粒子が存在することが判明した。さらに、このHA陽性で感染性がないウイルス液を免疫電顕で調べたところ、ウイルス粒子が確認され、ビリオンのエンベロープは金コロイド標識したHN蛋白を認識する抗体では反応したが、金コロイド標識したF蛋白を認識する抗体では反応しなかった(図20)。こ

のことはF-lessのビリオンの存在を示し、F蛋白がなくてもHN蛋白単独でウイルスがビリオンとして形成されることが判明した。F単独でSeVビリオンが形成できることはすでに報告されおり (Leyer, S. et al., J Gen. Virol 79, 683-687 (1998))、今回の結果はHN蛋白単独にSeVビリオンを形成できることが初めて明らかとなった。このようなF-lessビリオンを発育鶏卵で一過的に大量調製できることは、SeV F欠損RNPを包むビリオンを大量に生産ができることを示している。

### <3>

前述したように発育鶏卵で一過的に増幅されたF-lessウイルスビリオンはセンダイウイルスが感染可能な細胞にはまったく感染性を示さない。そこで機能的なRNP構造はエンベロープに包まれていることを確認するために、カチオニックリボソーム(DOSPER, Boehringer mannheim)と混合して、室温で15分間インキュベーションしてF発現細胞と非発現細胞にトランスフェクションした。その結果、カチオニックリボソームと混合しない場合は全くGFP発現細胞が観察されなかったのに対し、カチオニックリボソームと混合した場合はGFPの発現がいずれの細胞においても観察された。F非発現細胞ではGFPは単細胞で発現し、隣細胞に広がらないのに対し、F発現細胞ではGFP発現細胞は広がって、コロニーが形成されることが観察された(図21)。このことから、発育鶏卵で一過的に増幅された感染性のないビリオンはトランスフェクションなどの方法を用いて細胞に導入すれば、遺伝子を発現しうるということが明らかとなった。

### [実施例8] FHN欠損SeVゲノムからウイルスの再構築および増幅

#### <FHN欠損ゲノムcDNAの構築>

FHN欠失型SeVゲノムcDNA (pSeV18<sup>+</sup>/ΔFHN) の構築はまずpUC18/KSをEcoRIで消化してpUC18/Ecoを構築し、F遺伝子の開始コドンからHN遺伝子の終止コドンまでの間の全配列を(4866-8419)を欠失させ、BsiwI部位(cgtacg)で連結し構築した。FHN欠損部位の配列をシーケンスで確認した後、EcoRIフラグメント(4057bp)をゲルから回収してpUC18/KSのEcoRIフラグメントと置き換えて構築した。このFHN

欠損領域を含むKpnI/SphIフラグメント(14673bp)をゲル回収して pSeV18<sup>+</sup>の KpnI/SphIフラグメントと置き換え、プラスミド pSeV18<sup>+</sup>/ΔFHN が得られた。

一方、GFPを導入したFHN欠損SeV cDNAの構築は次のように行った。pSeV18<sup>+</sup>/ΔFHN からSalI/XhoIフラグメント(7842bp)を回収してpGEM11Z(Promega)にクロニングし、プラスミドpGEM11Z/SXdFHNとした。FHN欠失部位にd2EGFP (Clontech) の ATG-TAA (846 bp) の両端にBsiwI部位を付加したPCR産物をBsiwI酵素で消化して、pGEM11Z/SXdFHNのFHN欠損部位のBsiwI部位に連結した。得られたプラスミドは pSeV18<sup>+</sup>/ΔFHN-d2GFP とした。

#### <FHN欠損蛋白共発現細胞の作成>

F遺伝子を発現するプラスミドは前述したF欠損蛋白発現細胞株の作製に用いたものと同じのもので、HN遺伝子を発現するプラスミドはそれと同様な方法で構築し、HNのORFを含むフラグメントをpCALNdLw (Araiら,前記)のユニークなSwaI 部位に挿入し、プラスミドpCALNdLw/HNとした。

LLC-MK2細胞にpCALNdLw/FとpCALNdLw/HNを同量または異なる量比で混合し、mammalian transfection kit (Stratagene) を用いそのプロトコールに従って遺伝子導入を行った。G418で3週間選択した後クロニングした。得られた薬剤耐性クローンはそれぞれCre DNA レコンビナーゼを発現する組換えアデノウイルス (Ade/Cre) (齊藤ら, 前記) で感染し (moi=10)、FとHN蛋白質の誘導発現3日後細胞をPBS(-)で3回洗浄して回収し、ウェスタンブロッティング法を用いて抗SeV Fと抗SeV HN蛋白質のモノクローナルIgGにより検出した(図22)。

#### <pGEM/FHNの構築>

pCALNdLw/FとpCALNdLw/HNを構築に用いたFとHNフラグメントをそれぞれpGEM4Z、pGEM3Z (Promega社) にクロニングし、pGEM4Z/FとpGEM3Z/HNを得た。pGEM3Z/HNのT7プロモーターとHNを含む領域をPvuII酵素で消化して得られたフラグメントを回収し、pGEM4Z/FのF遺伝子下流のSacIユニークサイトで切断し平端化した部位にライゲーションした。F遺伝子とHN遺伝子を同一方向に並べたものは、抗Fまた

は抗HNモノクローナル抗体でウェスタンブロッティングを行い、FとHN両方の蛋白質は同時に発現できることを確認した。

#### <FHN欠損ウイルスの再構築>

FHN欠損ウイルスの再構築 (P0) は二通りに行った。一つはF欠損ウイルスの再構築と同様にRNPトランスフェクション法を用いた。もう一つはT7でFHN蛋白を共発現プラスミドを供給して再構築を行った。すなわち、T7プロモーターの制御下でF、HNタンパク質を発現するプラスミドを別途作製して、これによりFおよびHNタンパク質を供給して再構築を行った。いずれの方法においても再構築したものはFHN共発現細胞で増幅を行った。FHN欠損GFP発現SeV cDNA (pSeV18'/ $\Delta$ FHN-d2GFP), pGEM/NP, pGEM/P, pGEM/L, pGEM/FHNをそれぞれ、12 $\mu$ g/10cm dish, 4 $\mu$ g/10cm dish, 2 $\mu$ g/10cm dish, 4 $\mu$ g/10cm dish, 4 $\mu$ g/10cm dishの量比で混合し (最終容量, 3ml/10cm dish)、前述したF欠損SeVの再構築と同様な方法でLLC-MK2細胞に遺伝子導入した。遺伝子導入3時間後培地をAraC(40 $\mu$ g/ml, SIGMA), トリプシン (7.5 $\mu$ g/ml, GIBCO)入りのMEMに交換し、さらに3日間培養した。遺伝子導入後2日目で蛍光実体顕微鏡で観察し、pGEM/FHNの添加の有無の違いを検証し、GFP発現細胞の広がり度でウイルスの形成を確認した。その結果、再構築時にpGEM/FHNを添加した場合はGFP発現細胞の広がりが確認され、pGEM/FHNの添加がない場合はGFP発現はシングル細胞でしか観察されなかった (図23)。FHN蛋白再構築時に添加することでウイルスのビリオンが形成されたことを示した。一方、RNPトランスフェクションの場合はF欠損と同様にP1のFHN発現細胞でウイルスの回収に成功した (図24上)。

Ade/Creを感染して6時間以後にFHN蛋白が誘導発現された細胞にFHN欠損ウイルス液を感染し増幅ができたことを確認した (図24下)。

FHN欠損GFPを発現するcDNAから再構築されたウイルス液はLLC-MK2, LLC-MK2/F, LLC-MK2/HN, LLC-MK2/FHNに感染してトリプシンの添加の有無で培養した。培養3日後にGFP蛋白発現細胞の広がりを確認したところ、LLC-MK2/FHNでのみ

GFPの広がりを観察され、このウイルス液はFHN共発現に特異的かつトリブシン依存的に増幅されることが確認された(図25)。

FHN欠損ウイルスゲノムを確認するため、LLC-MK2/FHN細胞から回収された培養上清を遠心した後、QIAamp Viral RNA mini kit (QIAGEN)でそのプロトコールに従ってRNA抽出を行った。このRNAをSuperscript Preamplification System for first Strand Synthesis (GIBCO BRL)によりRT-PCRのテンプレート合成を行い、TAKARA Z-Taq(宝酒造)を用いてPCRを行った。対照群はF欠損ウイルスを用いた。PCRプライマーはM遺伝子とGFP遺伝子の組み合わせ、またはM遺伝子とL遺伝子の組み合わせを用いて行った(M遺伝子とGFP遺伝子の組み合わせ(M-GFP)については forward: 5'-atcagagacctgcgacaatgc/配列番号: 13, reverse: 5'-aagtcgtgctgcttcatgtgg/配列番号: 14; M遺伝子とL遺伝子の組み合わせ(M-L)については forward: 5'-gaaaaacttagggataaagtccc/配列番号: 15, reverse: 5'-gttatctccgggatggtgc/配列番号: 16)。その結果、MとGFP遺伝子をプライマーに用いた場合はRT条件下でF欠損とFHNとも欠損ウイルス共に特異的なバンドが検出された。MとL遺伝子をプライマーに用いた場合は、FHN欠損はGFPを含んだ所定のサイズのバンドが検出され、F欠損の場合はHN遺伝子を含んだサイズで長くなったバンドが観察された。ゲノム構造はFHN欠損していることは明らかとなった(図26)。

一方、FHN欠損ウイルスをF発現細胞にF欠損と同様に感染して、培養して4日目培養上清を回収し、LLC-MK2, LLC-MK2/F, LLC-MK2/FHNへの感染実験を行った。その結果、いずれの感染細胞においてもGFP発現細胞が観察されず、これらの細胞への感染性がないことを示した。しかし、F蛋白単独でウイルス粒子を形成できることがすでに報告され(Kato, A. et al., Genes cells 1, 569-579 (1996))、肝臓にあるアジアロ糖蛋白リセプター(ASG-R)を介して肝細胞に特異的に感染できることが報告された(SpiegelらJ. Virol 72, 5296-5302, 1998)。従って、FHN欠損RNAゲノムを持ち、ウイルスエンベロープはF蛋白のみで形成されたビリオンがF

発現細胞の培養上清に放出されることが考えられる。そこで、FHN欠損ウイルスを感染した F 発現細胞の培養上清を回収し、遠心した後上記の方法と同様に RNA 抽出を行い、前述した方法と同様に RT-PCR で解析した。その結果、図 27 で示したように FHN 欠損ゲノムを含む RNA が存在することが判明した。

この他、VSV-G とシュードタイプ化したウイルスのビリオンのウェスタンブロッティングによる解析では、F、HN 蛋白が発現していないことは明らかである。FHN 欠損ウイルスビリオンの生産系が確立したと言える。

さらに、F 蛋白発現細胞から放出されたビリオンをカチオニックリポソーム (50  $\mu$ l の DOSPER/500  $\mu$ l/well) との混合の有無で FHN 発現細胞または非発現 LLC-MK2 細胞に重層した。その結果、前述した F-less 粒子の場合と同様、DOSPER と混合して細胞に重層した場合には GFP 発現細胞の広がりが見られるが、HN-less のビリオンのみでは全く細胞に感染性なく、GFP 発現細胞が見られなかった。FHN 非発現細胞では GFP 発現細胞が見られたが、ウイルスが再形成され広がったことが認められなかった。

このような F 発現細胞から回収されるウイルス様粒子が ASG-R 遺伝子を持続発現する細胞株や非発現細胞株、または肝細胞に重層して感染し、Spiegel らの方法で肝臓特異的、または ASG-R に特異的に感染するかを調べることができる。

#### 【実施例 9】 欠損ゲノム RNA ウイルスベクターの応用性

1. 上に述べた系で増幅された F 欠損 RNP は F-less のウイルスエンベロープに包まれており、このエンベロープを化学的に修飾法などにより所望の細胞導入能を付加し、または遺伝子導入試薬や遺伝子銃のようなもので細胞に導入して (RNP トランスフェクション、または RNP インジェクション)、その組み換え RNA ゲノムが導入細胞で自律的に RNA 複製または蛋白を生産し続けることが可能である。
2. HN の細胞内ドメインを残し、細胞外ドメインを他のレセプターを特異的に標的できるリガンドを融合させ、キメラ蛋白を生産できる組み換え遺伝子をウイルスゲノムに組み込めば、特異性のある標的できるベクターの生産が可能となり、

また、この組み換え蛋白の生産細胞でベクターを調製可能である。これらのベクターは遺伝子治療、ワクチンなどに応用可能である。

3. FHNとも欠損するSeVウイルスの再構築に成功したことから、GFP遺伝子の代わりにターゲッティング可能なエンベロープキメラ蛋白の遺伝子をFHN欠損部位に導入し、FHN欠損ベクターと同様な方法で再構築し、FHN発現細胞で一度増幅して、非発現細胞に感染して、ウイルスゲノムから転写されたターゲッティング可能なキメラエンベロープ蛋白のみによって形成されたビリオンを回収すれば、ターゲッティングベクターの生産が可能となる。

4. これまでに、センダイウイルスのミニゲノム、NP,P,LとF遺伝子で細胞に同時に遺伝子導入してミニゲノムを包むF蛋白単独で形成されたビリオンが報告され (Leyerら, J Gen.Virol 79,683-687, 1998) また、マウスの白血病ウイルスをセンダイF蛋白でシュード化したベクターも報告されている (Spiegelら J. Virol 72, 5296-5302, 1998)。また、F蛋白質がトリプシンで解裂された後ASG-Rを介して肝臓細胞に特異的にターゲッティングできると報告されている (Bitzerら J.Virol.71, 5481-5486, 1997)。前の報告の系は一過的な粒子形成系であり、持続的にベクターの粒子回収が困難である。またSpiegelらはセンダイF蛋白でシュードタイプ化したレトロウイルスベクターを報告しているが、レトロウイルスは分裂細胞にしか遺伝子導入できないなどの固有の問題を抱えている。本発明で回収された、FHN共欠損SeVウイルスゲノムを持ち、F蛋白のみエンベロープ蛋白を持つウイルス粒子等は、細胞分裂に関係なく効率的な細胞質で自律複製可能なRNAベクターであり、新規のウイルス粒子であり、またその大量生産が可能な実用的な系である。

#### 〔実施例10〕 FHN欠損SeVゲノムからウイルスの再構築および増幅

センダイウイルス、麻疹ウイルス等の多くの一本鎖マイナス鎖RNAウイルスで、ウイルスゲノムをクローニングしたcDNAから感染可能なウイルス粒子を再構成する技術が確立された。



ほとんどの系で、T7プロモーター下流にcDNA、NP,P,L遺伝子を導入したプラスミドを細胞内に導入し、T7ポリメラーゼを用いてcDNA、各遺伝子を発現させることにより再構成を行っているが、T7ポリメラーゼの供給には、T7ポリメラーゼ発現組み換えワクシニアウイルスが主に使われている。

T7発現ワクシニアウイルスは、ほぼすべての細胞に効率よくT7ポリメラーゼを発現させることができるが、ワクシニアウイルス由来の細胞障害性のために、感染細胞を2、3日しか生存させることができない。多くの場合、抗ワクシニア薬剤としてリファンピシンを用いているが、加藤らの系(Kato, A. et al., Genes cells 1, 569-579 (1996))では、リファンピシンのほかに、AraCを並行して用いることにより、ワクシニアウイルスの増殖を最小限に抑え、センダイウイルスの再構成を効率よく行うことに成功した。

しかしながら、センダイウイルスを始めとするマイナス鎖RNAウイルスの再構成は $1 \times 10^5$ 細胞中に再構成されたウイルスが数粒子かそれ以下という効率で、レトロウイルス等のほかのウイルスに比べるといまだにかなり低いのが実情である。この理由として、ウイルス自体が持つ、再構成までの複雑な過程(裸のRNAに別途転写、翻訳されたタンパク質がついてRNP様構造となり、その後、ポリメラーゼにより転写、複製が行われる)とともに、ワクシニアウイルスを用いることによる細胞障害性も挙げられる。

T7ポリメラーゼを供給する手段として、ワクシニア以外にアデノウイルスの系も試みたが、よい結果は得られなかった。ワクシニアウイルスはT7ポリメラーゼのほかに細胞質で働くRNAキャッピング酵素も自身の蛋白としてコードしており、この酵素が、細胞質でT7プロモーターにより転写されたRNAをキャッピングして安定化することにより翻訳効率を高めていると考えられる。本発明では、ワクシニアウイルスをPsoralen-Long-Wave-UV法で処理することにより、ワクシニアウイルスに由来する細胞障害を回避し、センダイウイルスの再構成効率を高めることを試みた。

ソラレンと長波長紫外線によるDNAクロスリンキングにより、DNAをゲノムに持つウイルスの複製を阻害し、しかし特に初期遺伝子の発現には影響を与えない状態を作り出すことが可能である。ワクシニアウイルスはゲノム長が長いので、この系によるウイルスの不活化の影響が顕著にあらわれると考えられる (Tsung, K. et al., J Virol 70,165-171 (1996))。

自立増殖可能な野生型ウイルスの場合、再構成により一粒子でもウイルスができていればトランスフェクションした細胞を発育鶏卵に接種してセンダイウイルスを増殖させることが可能なため、再構成の効率、そしてワクシニアウイルスの残留にそれほど気を使わなくてもよい。

しかし、ウイルスの複製、粒子形成の機構などを調べるために作る様々な変異ウイルスの再構成では、増殖に発育鶏卵を使用できずにウイルス由来のタンパク質を発現している細胞株などを用いざるを得ない場合もありうる。また、変異ウイルスまたは欠損ウイルスが野生型ウイルスに比べて顕著に増殖が遅いケースも、十分考えられる。

こうした変異を持つセンダイウイルスを増殖させるためには、トランスフェクション後の細胞を次代の細胞に重層して長時間培養しなければならない。そのために、再構成の効率とワクシニアウイルスの残存タイターが問題になってくる。本方法では、再構成効率を上昇させるとともに、残存ワクシニアウイルスのタイターを減少させることもできた。

本方法を用いて、現在までの、未処理のワクシニアウイルスを用いた系では得られなかった変異ウイルスを再構成によりうることができた (F、F H N 欠損ウイルス)。この系は、今後増えるであろう変異ウイルスの再構成に大きなツールとなると考える。そこで本発明者らは、ソラーレンと紫外線 (UV) の量を検討し、ワクシニアウイルスの不活化の条件を検討した。

#### <実験>

まず、照射時間を二分間に定め、ソラレン濃度の検定を行った。不活化の検定

は、ブランク形成によるワクシニアウイルスのタイターの測定と、T7プロモーター支配下pGEM-luciプラスミド、センダイウイルスミニゲノムによるT7ポリメラーゼ活性の測定によって行った。センダイウイルスミニゲノムによるT7ポリメラーゼ活性の測定はセンダイウイルスミニゲノムのプラスミドと、T7でセンダイウイルスNP、P、L蛋白を発現するpGEM/NP, pGEM/P, pGEM/Lプラスミドと同時に細胞にトランスフェクションし、リボヌクレオ蛋白複合体を形成させ、センダイウイルスのRNAポリメラーゼによりルシフェラーゼ酵素蛋白の転写を調べる系である。

UV照射二分間では、ソラレンの濃度に応じてワクシニアウイルスのタイターの減少が見られた。しかし、T7ポリメラーゼ活性は、ソラレン濃度が0、0.3、1  $\mu$ g/ml迄は変化を見せず、10  $\mu$ g/mlでは 10分の1 程度に減少していた。(図 2 8)。

さらに、ソラレン濃度を0.3  $\mu$ g/mlに固定し、紫外線照射時間を検討した。照射時間が増大するに連れ、ワクシニアウイルスのタイターは減少したが、30分までの照射ではT7ポリメラーゼ活性への影響は見られなかった。このとき、0.3  $\mu$ g/ml、30分照射の条件では、T7ポリメラーゼ活性に影響を与えず、タイターを1000分の1にまで減少させることができた。(図 2 9)

しかしながら、タイターが1000分の1にまで減少したワクシニアウイルスでも処理前のタイターに換算してmoi=2 (処理後の残存タイターでmoi=0.002) で感染したときの24時間後のCPEは、未処理のウイルスをmoi=2で感染させたときのそれと変わらなかった (図 3 0)。

この条件で処理したワクシニアウイルスを用い、センダイウイルス再構成の効率を検討した。再構成は、前記加藤らの方法をモディファイし、以下の手順で行った。6wellのマイクロプレートにLLC-MK2細胞を $3 \times 10^5$ 細胞/wellで撒き、終夜培養した後、PLWUV処理前のタイター換算で $6 \times 10^5$  pfu/100  $\mu$ lとなるようにワクシニアウイルスを希釈し、PBS洗浄後の細胞に感染させた。1時間の感染後、100  $\mu$ lのOPTI-MEMにプラスミド、pGEM-NP, P, L、そしてcDNAをそれぞれ1, 0.5, 1

、4 $\mu$ gを加えたものに、Superfect (QIAGEN) を10 $\mu$ l加え、室温で15分放置した後1mlのOPTI-MEM (GIBCO) (Rif. AraCを含む) をくわえ、細胞に重層した。

トランスフェクション後2、3、4日目に細胞を回収し、遠心後、300 $\mu$ l/wellのPBSに懸濁した。この懸濁液を原液、あるいは10倍、100倍希釈した細胞溶液100 $\mu$ lを受精後10日目の発育鶏卵に各希釈4個ずつ接種した(1 $\times$ 10<sup>5</sup>, 1 $\times$ 10<sup>4</sup>, 1 $\times$ 10<sup>3</sup>細胞をそれぞれ接種)。3日後鶏卵から尿液を回収しHA試験によりウイルス再構成の有無を調べた(表1)。1 $\times$ 10<sup>5</sup>細胞を接種した鶏卵のうち、HA活性があった鶏卵を一点、10<sup>4</sup>では十点、10<sup>3</sup>では百点と数えて、再構成の効率(Reconstitute Score)を求めた(図31)。計算式は表1の通り。

表1 ワクシニアウイルスのUV処理時間のセンダイウイルス再構成効率に対する影響

接種細胞数	スコア - (a)	HA陽性卵数 (b)											
		2d						3d					
		0'	15'	20'	30'	0'	15'	20'	30'	0'	15'	20'	30'
$10^5$	1 (a1)	1	2	4	4	0	2	4	4	1	3	4	4
$10^4$	10 (a2)	0	1	3	2	0	2	3	4	0	0	4	0
$10^3$	100 (a3)	0	0	0	1	0	1	0	2	0	0	0	0
Reconsti- tution Score	(a1+a2+a3) x b	1	12	24	124	0	122	34	244	1	3	44	4

Reconstitution Score = (a1+a2+a3) x b

また、トランスフェクション後2、3、4日での、細胞に残存するワクシニアウイルスのタイターを測ったところ、トランスフェクション前に与えたタイターに比例して、処理をしたものが少なくなっていた (図3 2)。

ワクシニアウイルスをPLWUVで不活化することにより、T7ポリメラーゼ活性には影響を与えず、タイターを1000分の1にまで下げることができた。しかし、ワクシニアウイルス由来のCPEは、顕微鏡観察で未処理の、1000倍のタイターを持つウイルスのそれと変わらなかった。

この条件で処理をしたワクシニアウイルスを、センダイウイルス再構成に用いることにより、センダイウイルスの再構成効率が、数十倍から百倍ほど増大した(図31)。同時に、トランスフェクション後に残ったワクシニアウイルスのタイターは、 $5\text{pfu}/10^5\text{ cells}$ 以上ではなかった。従って、複製可能なワクシニアウイルスの残留は0.005%以下に抑えられた。

[実施例11] シュードタイプセンダイウイルスの作製

<1> VSV-G 遺伝子産物を誘導発現するヘルパー細胞の作製

VSV-G 遺伝子産物は細胞障害性を有しているため、Cre リコンビナーゼにより VSV-G 遺伝子産物が誘導発現されるよう設計されたプラスミド pCALNdLG (Arai T. ら J.Virology 72 (1998) p1115-1121) を用い、LLC-MK2 細胞での安定導入株の作出を行った。LLC-MK2 細胞へのプラスミドの導入は、リン酸カルシウム法 (CalPhosTM Mammalian Transfection Kit、クローンテック社製) により、添付マニュアルに従って行った。

10 cm プレートを用い、60%コンフルエントまで生育した LLC-MK2 細胞に10  $\mu\text{g}$  のプラスミド pCALNdLG を導入後、10 ml の MEM-FCS10% 培地にて、37°Cの5%  $\text{CO}_2$  インキュベーター中で24時間培養した。24時間後に細胞を剥がし、10 ml の培地に懸濁後、10 cm シャーレ5枚を用い5 ml 1枚、2 ml 2枚、0.5 ml 2枚に撹き、G418 (GIBCO-BRL社製) 1200  $\mu\text{g}/\text{ml}$  を含む10 ml の MEM-FCS10% 培地で培養を行い、2日毎に培地交換しながら、14日間培養し、遺伝子の安定導入株の選択を行った。該培養により生育してきたG418に耐性を示す細胞は、クローニングリングを用いて28株を回収した。各クローンは10 cm プレートでコンフルエントになるまで拡大培養を続けた。

各クローンについて、Cre リコンビナーゼを含む組み換えアデノウイルス AxCANCreを感染後、抗 VSV-G モノクローナル抗体を用いて、VSV-G の発現を以下に記載のウエスタンブロット法により調べた。

各クローンは6 cm シャーレにて、コンフルエントまで生育させた後、アデノ

ウイルス AxCANCre を齋藤らの方法（上記）により MOI=10で感染後、3日間培養した。該細胞は培養上清を取り除いた後、PBS緩衝液で洗浄し、0.05% トリプシン、0.02% EDTA(エチレンジアミン4酢酸)を含むPBS緩衝液 0.5 mlを加え、37℃、5分間インキュベートすることによりシャーレより剥がした。該細胞は3 ml PBS緩衝液に懸濁後、1,500 x gで5分間遠心し、細胞を集めた。得られた細胞はさらに2ml PBS緩衝液に再度懸濁後、1,500 x gで5分間遠心分離することにより、細胞を集めた。

該細胞は-20℃で保存することが可能で、必要に応じて解凍して使用することができる。集めた細胞は100  $\mu$ L の細胞溶解液（RIPA バッファー、ベーリンガーマンハイム社製）により溶解し、該細胞の全蛋白質（1レーン当たり  $1 \times 10^5$ 細胞）を用いてウェスタンブロットを行った。細胞溶解液をSDS-ポリアクリルアミドゲル電気泳動用サンプルバッファー〔6mM トリス-塩酸（pH6.8）、2% SDS、10% グリセロール、5% 2-メルカプトエタノールからなる緩衝液〕に溶解し、95℃5分加熱後電気泳動用試料に供した。該試料をSDS-ポリアクリルアミドゲル（マルチゲル 10/20、第一化学社製）を用い、電気泳動により分画し、分画された蛋白質をセミドライブロット法により転写膜（Immobilon-P TransferMembranes、Millipore 社製）に転写した。転写は、100% メタノールに20秒、水に一時間浸した転写膜を使用し、1mA/cm<sup>2</sup>定電流の条件で1時間行った。

該転写膜を、40 ml のブロッキング溶液（ブロックエース、雪印社製）中で1時間振盪させた後、PBS緩衝液で一度洗浄した。

該転写膜および10%ブロッキング溶液を含むPBS緩衝液で1/1000に希釈した抗 VSV-G 抗体（クローン P4D4、シグマ社製）5 ml をビニールバッグに入れてシールし、4℃で静置させた。

該転写膜を2度40 ml のPBS-0.1% Tween 20に5分間浸漬し、洗浄した後、PBS緩衝液で5分間浸漬し、洗浄した。

- 58 -

該転写膜および10%ブロッキング溶液を含むPBS緩衝液で1/2500に希釈したパーオキシダーゼで標識された抗マウスIgG抗体 (anti-mouseimmunoglobulin, Amersham 社製) 5 mlをビニールバッグに入れ、シールをした後、室温で1時間振盪させた。

振盪後、該転写膜を2度PBS-0.1%Tween 20に5分間浸漬し、洗浄した後、PBS緩衝液に5分間浸漬し、洗浄した。

発光法 (ECL Western blotting detection reagents, Amersham 社製) により、抗VSV-G抗体と交叉の見られる該転写膜上の蛋白質の検出を行った。結果を図33に示す。3クローンで、AxCANCre 感染特異的な VSV-G の発現が検出され、VSV-G 遺伝子産物を誘導発現するLLC-MK2 細胞の作出が確認された。

得られた細胞株の一株をLLCG-L1と呼び、抗VSV抗体を用いてフローサイトメトリ解析を行った (図34)。その結果、LLCG-L1では、VSV-G 遺伝子誘導発現時特異的に抗体との反応性が検出され、VSV-G タンパク質が細胞表面に発現されることが確認された。

<2> ヘルパー細胞を用いたF 遺伝子を欠失したゲノムを有するシュードタイプセンダイウイルスの作製

F 遺伝子を欠失したゲノムを有するセンダイウイルスを VSV-G遺伝子発現細胞に感染させ、VSV-Gを外被に有するシュードタイプウイルスの産生が見られるかを、上記実施例に記載のGFP遺伝子を含むF 欠失型センダイウイルスを用い、GFP遺伝子の発現を指標に調べた。その結果、Cre リコンビナーゼを含む組み換えアデノウイルスAxCANCre を感染しないLLCG-L1では、F 欠失型センダイウイルスの感染によりウイルス遺伝子が導入され、GFP発現細胞は検出されるものの、その細胞数は増えず、VSV-Gを誘導発現させた細胞では、経時的にGFP発現細胞の増加が認められた。その上清の1/5量をさらに、新たなVSV-G を誘導発現させた細胞に添加したところ、前者由来の上清では、遺伝子導入が全くみとめられず、後者由来の上清では遺伝子導入およびGFP発現細胞の増加が認められた。また、後者由来の



上清をVSV-Gを誘導しないLLCG-L1細胞に添加した際には、遺伝子導入はされるものの、GFP発現細胞の増加は認められなかった。以上の結果から、VSV-G発現細胞特異的にウイルスが増殖することが認められ、VSV-GとのシュードタイプのF欠失型センダイウイルスの生成が認められた。

#### <3> F 遺伝子を欠失したゲノムを有するシュードタイプセンダイウイルスの産生条件の検討

VSV-G 遺伝子の発現量の影響を調べるため、AxCANCRe の感染量 (MOI=0、1、2.5、5、10) を変え、一定量のF 遺伝子を欠失したゲノムを有するシュードタイプセンダイウイルスを感染後、7日目から8日目の上清を回収し、さらにVSV-G 誘導前、誘導後の細胞に感染させ、5日目のGFP の発現している細胞の数を比較したところ、MOI=0 ではウイルスの産生が全く認められず、MOI=10の条件で最も多いことがわかった (図35)。また、経時的にウイルス産生量を調べたところ、シュードタイプセンダイウイルス感染後5日目以降から産生量が上昇し、8日目まで産生が確認できた (図36)。ウイルス力価の測定は、VSV-G 誘導前の細胞に、10倍ずつ段階的に希釈したウイルス液を添加し、感染後5日目のGFP の発現細胞を数えることにより、ウイルス液中の細胞への感染粒子数 (CIU) を求めた。その結果、最高ウイルス産生量は  $5 \times 10^5$  CIU/mlであった。

#### <4> F 遺伝子を欠失したゲノムを有するシュードタイプセンダイウイルスの抗VSV抗体による感染性の影響

VSV-G 発現株を用いて得られたF 遺伝子を欠失したゲノムを有するシュードタイプセンダイウイルスが、外被にVSV-G タンパク質を有するかに関して、抗VSV抗体を用いて感染性の影響されるかどうかの中和活性を調べた。ウイルス液と抗体を混合し、室温で30分静置後、VSV-Gを誘導発現していないLLCG-L1細胞に感染し、5日目の遺伝子導入能をGFP発現細胞の有無で調べた。その結果、抗VSV抗体で感染性の完全な抑制が認められ、本来の外被を有するF 遺伝子を欠失したゲノムを有するセンダイウイルスでは抑制が認められなかった (図37)。このこ

- 60 -

とから、今回得られたウイルスが、外被にVSV-G タンパク質を有するシュードタイプのセンダイウイルスであり、抗体によりその感染性が特異的に抑えられることが明らかとなった。

〈5〉 シュードタイプセンダイウイルスが F 欠失型ゲノムを有することの確認  
今回VSV-G 遺伝子発現細胞で増殖したウイルスが F 欠失型であることを、感染細胞抽出液のタンパク質のウェスタン解析により調べた。ウェスタン解析は、上記に記載の方法により行った。一次抗体として、ウサギより調製された、抗センダイウイルスポリクローナル抗体、マウスより調製された抗Fタンパク質モノクローナル抗体、マウスより調製された抗HNタンパク質モノクローナル抗体を用い、2次抗体に、抗センダイウイルスポリクローナル抗体の場合はパーオキシダーゼで標識された抗ウサギ Ig G抗体、抗Fタンパク質モノクローナル抗体、抗HNタンパク質モノクローナル抗体の場合はパーオキシダーゼで標識された抗マウス Ig G抗体を用いた。その結果、センダイウイルス由来のタンパク質およびHNタンパク質は検出されるものの、Fタンパク質は検出されなかったことから、F欠失型であることが確認された。

〈6〉 ヘルパー細胞を用いたFおよびHN 遺伝子を欠失したゲノムを有するシュードタイプセンダイウイルスの作製

Fおよび HN 遺伝子を欠失したゲノムを有するセンダイウイルスを VSV-G遺伝子発現細胞LLCG-L1に感染させ、VSV-Gを外被に有するシュードタイプウイルスの産生が見られるかを、上記実施例に記載のGFP遺伝子を含むF、HN 欠失型センダイウイルスを用い、上記実施例と同様の方法で GFP遺伝子の発現を指標に調べた。その結果、VSV-G 発現細胞特異的にウイルスが増殖することが認められ、VSV-G とのシュードタイプのF、HN欠失型センダイウイルスの生成が認められた（図38）。ウイルス力価の測定は、VSV-G 誘導前の細胞に、10倍ずつ段階的に希釈したウイルス液を添加し、感染後5日目のGFPの発現細胞を数えることにより、ウイルス液中の細胞への感染粒子数（CIU）を求めた。その結果、最高ウイルス産生量は

$1 \times 10^6$  CIU/mlであった

。

<7> シュードタイプセンダイウイルスが Fおよび HN 欠失型ゲノムを有することの確認

今回VSV-G 遺伝子発現細胞で増殖したウイルスが Fおよび HN 欠失型であることを、感染細胞抽出液のタンパク質のウェスタン解析により調べた。その結果、センダイウイルス由来のタンパク質は検出されるものの、FおよびHNタンパク質は検出されなかったことから、Fおよび HN欠失型であることが確認された（図39）。

#### 【実施例12】 ウイルス再構成法の検討

##### <従来法>

LLC-MK2 細胞を  $5 \times 10^6$  cells/dish で100 mm ペトリ皿に蒔き、24 時間培養後、血清を含まないMEM培地で 1 回洗浄した後、 $3 \mu\text{g/ml}$  のソラレンと長波長紫外線 (365nm) で 5 分間処理した T7 RNA ポリメラーゼを発現するリコンビナントワクシニアウイルス (Fuerst, T.R. et al., Proc.Natl.Acad.Sci.USA 83, 8122-8126 1986)(vTF7-3)に室温で 1 時間感染させた (moi=2) (moi=2~3、好適には moi=2 が用いられる)。細胞を、血清を含まないMEM培地で 2 回洗浄した後、プラスミド pSeV18'/ $\Delta$ F-GFP, pGEM/NP, pGEM/P, 及びpGEM/L(Kato, A. et al., Genes cells 1, 569-579(1996)) をそれぞれ  $12 \mu\text{g}$ ,  $4 \mu\text{g}$ ,  $2 \mu\text{g}$ , 及び  $4 \mu\text{g/dish}$  の量比で Opti-MEM培地(GIBCO)に懸濁し、SuperFect transfection reagent ( $1 \mu\text{g DNA}/5 \mu\text{l}$  のSuperFect, QIAGEN)を入れ、室温で 15 分間放置後、最終的に 3% FBSを含む Opti-MEM培地 3 ml に入れた DNA-SuperFect 混合物を細胞に添加して培養した。3時間培養後、細胞を、血清を含まないMEM培地で 2 回洗浄し、シトシン $\beta$ -D-アラビノフラノシド  $40 \mu\text{g/ml}$  (AraC, Sigma)を含むMEM培地で 70 時間培養した。これらの細胞と上清を回収し、それぞれ P0-d3 サンプルとした。P0-d3 のペレットを Opti-MEM培地に懸濁した ( $10^7$  cells/ml)。凍結融解を 3 回繰

- 62 -

り返して lipofection reagent DOSPER (Boehringer mannheim)と混合し( $10^6$  cells/25 $\mu$ l DOSPER)室温で 15 分間放置した後、F発現 LLC-MK2/F7 細胞株にトランスフェクション( $10^6$  cells/well 24-well-plate)し、血清を含まないMEM培地(40 $\mu$ g/ml AraC, 7.5 $\mu$ g/ml トリプシンを含む)で培養した。培養後 3 日目および 7 日目に上清を回収し、それぞれ P1-d3 および P1-d7サンプルとした。

#### <エンベローププラスミド+F発現細胞重層法>

プラスミドにエンベローププラスミド pGEM/FHN を4 $\mu$ g/dish加えた以外は、上記と同様の操作を行い、トランスフェクションを行った。3 時間培養後、細胞を、血清を含まない MEM培地で 2 回洗浄し、シトシン $\beta$ -D-アラビノフラノシド 40  $\mu$ g/ml (AraC, Sigma) とトリプシン 7.5 $\mu$ g/ml を含むMEM培地で 48 時間培養した。培養上清を取り除き、血清を含まない MEM培地(40 $\mu$ g/ml AraC, 7.5 $\mu$ g/ml トリプシンを含む)に懸濁された100 mm ペトリ皿 1 枚分の F 発現 LLC-MK2/F7 細胞懸濁液 5 ml を重層した。培養48時間後、これらの細胞と上清を回収し、それぞれ P0-d4 サンプルとした。P0-d4 のペレットを Opti-MEM培地に懸濁し(2 $\times$   $10^7$ cells/ml)、凍結融解を 3 回繰り返して F 発現 LLC-MK2/F7 細胞株に重層(2 $\times$   $10^6$ cells/well 24-well-plate)し、血清を含まないMEM培地(40 $\mu$ g/ml AraC, 7.5  $\mu$ g/ml トリプシンを含む)で培養した。培養後 3 日目および 7 日目に上清を回収し、それぞれ P1-d3 および P1-d7サンプルとした。比較のため、重層を行わず、エンベローププラスミドのみを添加し、上記の従来法と全く同じ方法でも実験を行った。

#### <GFP発現細胞のカウントによるCIUの測定(GFP-CIU)>

LLC-MK2 細胞を 2 $\times$   $10^5$  cells/wellで12well-plateに蒔き、24 時間培養後、血清を含まない MEM培地で 1 回洗浄した後、上記のサンプル(P0-d3またはP0-d4、P1-d3 および P1-d7)を、陽性細胞が 10cm<sup>2</sup> 中に10~100個の間の数になるように適宜希釈し、100 $\mu$ l/wellで感染させた。15分後血清を含まない MEM培地を 1 ml/well 加えた。さらに24 時間培養後、細胞を蛍光顕微鏡下で観察し、GFP発現

細胞のカウントを行った。

<CIU(Cell-Infected Unit)測定>

LLC-MK2 細胞を  $2 \times 10^5$  cells/dish で12well-plate に蒔き、24 時間培養後、血清を含まない MEM培地 で 1 回洗浄した後、上記サンプル (含まれるウイルスベクターをSeV/ $\Delta$ F-GFPと称す) を  $100 \mu\text{l/well}$  で感染した。15分後、血清を含まない MEM培地を $1\text{ml/well}$  加え、さらに24 時間培養した。培養後、PBS(-)で 3 回洗浄した後、細胞を乾燥させ (約10分~15分室温放置)、細胞を固定するため、アセトンを  $1\text{ml/well}$  加え直ちにに取り除き、再び乾燥させた (約10分~15分室温放置)。PBS(-)で 100 倍希釈したウサギより調製された抗SeVポリクローナル抗体 (DN-1)を  $300 \mu\text{l/well}$  加え、 $37^\circ\text{C}$ で45分間インキュベートした後、PBS(-)で 3 回洗浄し、PBS(-)で 200 倍希釈した抗ウサギIgG(H+L)蛍光標識二次抗体 (AlexaTM568:Molecular Probes社製)を  $300 \mu\text{l/well}$  加え、 $37^\circ\text{C}$ で45分間インキュベートした。PBS(-)で 3 回洗浄した後、蛍光顕微鏡下(Emission:560nm , Absorption:645nmフィルター:ライカ社製)で蛍光を発する細胞を観察した (図 4 0)。

対照として上記サンプル (SeV/ $\Delta$ F-GFP) を  $100 \mu\text{l/well}$  で感染し15分後、血清を含まない MEMを $1\text{ml/well}$  加え、さらに24 時間培養後、以後の操作を行わずに細胞を蛍光顕微鏡下(Emission:360nm , Absorption:470nmフィルター:ライカ社製)でGFP発現細胞を観察した。

【実施例 1 3】 欠失型センダイウイルスベクターの再構成効率向上のための最適なワクシニアウイルス(vTF7-3)のPLWUV(Psoralen and Long-Wave UV Light)処理条件検討

LLC-MK2 細胞を  $5 \times 10^6$  cells/dish で100 mm ペトリ皿に蒔き、24 時間培養後、血清を含まない MEM培地 で 1 回洗浄した後、 $0.3 \sim 3 \mu\text{g/ml}$  のソラレンと長波長紫外線 (365nm) で 2~20 分間処理した T7 RNA ポリメラーゼを発現するリコンビナントワクシニアウイルス (vTF7-3) (Fuerst, T.R. et al.,

Proc.Natl.Acad.Sci.USA 83, 8122-8126(1986)に室温で 1 時間感染させた (moi=2) (moi=2~3、好適には moi=2 が用いられる)。細胞を血清を含まない MEM 培地で 2 回洗浄した後、プラスミド pSeV18'/ $\Delta$ F-GFP, pGEM/NP, pGEM/P, 及び pGEM/L(Kato, A. et al., Genes cells 1, 569-579(1996)), をそれぞれ 12 $\mu$ g, 4  $\mu$ g, 2 $\mu$ g, 及び 4 $\mu$ g/dish の量比で Opti-MEM培地(GIBCO)に懸濁し、SuperFect transfection reagent (1 $\mu$ g DNA/5 $\mu$ l のSuperFect, QIAGEN)を入れ、室温で 15 分間放置後、最終的に 3%FBSを含むOpti-MEM培地 3 ml に入れた DNA-SuperFect 混合物を細胞に添加して培養した。3 時間培養後、細胞を、血清を含まない MEM 培地 で 2 回洗浄し、シトシン $\beta$ -D-アラビノフラノシド 40  $\mu$ g/ml (AraC, Sigma)を含む MEM培地 で 48 時間培養した。100 mm ペトリ皿の約 1/20 視野の細胞を蛍光顕微鏡下で観察し、GFP発現細胞のカウントした。ワクシニアウイルス (vTF7-3)の不活化の検定にはプラーク形成によるタイターの測定(永井美之ら, ウイルス実験プロトコール, p291-296, 1995)を行った。

さらに、トランスフェクション後の回収時期を3日目に着目し、ソラレンとUV 照射時間の検討を行った。各PLWUV処理を行ったワクシニアウイルス (vTF7-3) を用い、センダイウイルス再構成の効率を検討した。再構成は加藤らの方法(上記)を改変し、以下の手順で行った。6 wellのマイクロプレートに LLC-MK2 細胞を  $5 \times 10^5$  細胞/wellで撒き、終夜培養した後 ( $1 \times 10^6$  細胞/wellに増殖していると仮定)、PLWUV処理前のタイター換算で  $2 \times 10^6$  pfu/100 $\mu$ l となるようにワクシニアウイルス(vTF7-3)を希釈し、PBS洗浄後の細胞に感染させた。1時間の感染後、50 $\mu$ l の Opti-MEM培地(GIBCO)にプラスミド、pGEM/NP, pGEM/P, 及びpGEM/L、そして付加型SeV cDNA (pSeV18' b(+)) (Hasan, M. K. et al., J. General Virology 78: 2813-2820, 1997) をそれぞれ 1, 0.5, 1, 4  $\mu$ gを加えたものに、SuperFect(QIAGEN)を 10 $\mu$ l 加え、室温で15分放置した後、1 mlの Opti-MEM(40  $\mu$ g/ml の AraC を含む)を加え、細胞に重層した。トランスフェクション後、3 日目に細胞を回収し、遠心後、100 $\mu$ l/wellのPBSに懸濁した。この懸濁液を10倍、

100倍、1000倍希釈した細胞溶液 100 $\mu$ l を受精後 10 日目の発育鶏卵に各希釈 3 個ずつ摂取した。(1 $\times$ 10<sup>5</sup>, 1 $\times$ 10<sup>4</sup>, 1 $\times$ 10<sup>3</sup> 細胞をそれぞれ摂取)。3 日後鶏卵から尿液を回収し HA 試験によりウイルス再構成の有無を調べた。1 $\times$ 10<sup>5</sup> 細胞を摂取した鶏卵のうち、HA 活性があった鶏卵を一点、10<sup>4</sup> では十点、10<sup>3</sup> では百点と数えて、再構成の効率を求めた。

#### <結果>

実施例 1 2 および 1 3 の結果を図 4 0 ~ 4 3、および表 2 に示す。エンベロープ発現プラスミドと細胞重層の組み合わせによる SeV/ $\Delta$ F-GFP の再構成効率の向上が確認された。P0 (継代前) の d3~d4 (3 日目~4 日目) において、著しい改善が認められた (図 4 1)。表 2 では、トランスフェクション後 3 日目の細胞を卵に接種した。0.3 $\mu$ g/ml のソラレン濃度で 20 分間の処理が、最も再構成効率が高かった (3 日目) ことから、この条件を最適条件とした (表 2)。

表2 センダイウイルス再構成に及ぼすワクシニアウイルスのPLWUV処理の影響

接種細胞数	スコア (a)	HA陽性卵数(b)							
		0 μg/ml	0.3 μg/ml	1 μg/ml			3 μg/ml		
		0'	20'	5'	10'	20'	2'	5'	10'
10 <sup>5</sup>	1(a1)	0	3	3	3	3	3	3	3
10 <sup>4</sup>	10(a2)	0	3	2	3	3	1	3	1
10 <sup>3</sup>	100(a3)	0	3	0	1	1	0	1	0
Reconsti- tution Score	(a1+a2+a3)×b	0	333	43	133	133	13	133	13
Reconstitution Score = (a1+a2+a3)×b									

センダイウイルスの再構成に及ぼすワクシニアウイルスのPLWUV処理の影響  
(トランスフェクション後3日目の細胞を卵に接種)

〔実施例14〕 GFPを含まないLacZ搭載 F 欠失型センダイウイルスベクターの  
作製

<LacZ 遺伝子を含む F 欠失型 SeV ベクター cDNA の構築>

実施例1記載の pSeV18'/ΔF のNP 遺伝子上流域に存在する Not I 切断部位に  
LacZ 遺伝子を搭載した cDNA (pSeV(+18:LacZ)/ΔF) を構築するため PCR により  
LacZ 遺伝子の増幅を行った。LacZ 遺伝子を6の倍数 (Hausmann, S et al., RNA  
2, 1033-1045 (1996)) にあわせ、5' 末側には NotI 切断部位を付与したプライ  
マー (5'-GCGCGGCCGCGTACGGTGGCAACCATGTCGTTTACTTTGACCAA-3' / 配列番号: 17  
) を、3' 末に SeV の転写終結シグナル (E)、介在配列 (I) および転写開始シグナ



- 67 -

ル (S) を付与し、NotI 切断部位を付与したプライマー (5'-GCGCGGCCGCGATGAACTTTCACCTAAGTTTTTCTTACTACGGCGTACGCTATTACTTCTGACACCAGACCAACTGGTA-3' / 配列番号: 18) を用い、プラスミド pCMV- $\beta$  (クローンテック社製) を鋳型としてPCR 反応を行った。反応条件は、pCMV- $\beta$  50 ng、200  $\mu$ M dNTP (ファルマシアバイオテク社製)、100 pM プライマー、Vent ポリメラーゼ (ニューイングランドバイオラボ社製) 4U を添付の反応バッファーとともに混合後、94°C 30 秒、50 °C 1 分、72 °C 2 分の反応温度サイクル25回で行った。反応産物をアガロースゲル電気泳動で泳動後、3.2 キロベースの断片を切り出し、精製後、NotI で切断し、pSeV18+/ $\Delta$ F Not I 切断片とライゲーションしてpSeV(+18:LacZ)/ $\Delta$ F を得た。

#### <従来法>

LLC-MK2 細胞を  $5 \times 10^6$  cells/dish で100 mm ペトリ皿に蒔き、24 時間培養後、血清を含まない MEM で 1 回洗浄した後、3  $\mu$ g/ml のソラレンと長波長紫外線 (365nm) で 5 分間処理した T7 RNA ポリメラーゼを発現するリコンビナントワクシニアウイルス(vTF7-3) (Fuerst, T.R. et al., Proc. Natl. Acad. Sci. USA 83, 8122-8126(1986))に室温で 1 時間感染させた (moi=2) (moi=2~3、好適には moi=2 が用いられる)。細胞を血清を含まない MEM で 2 回洗浄した後、LacZ 搭載 F 欠失型センダイウイルスベクター cDNA (pSeV (+18:LacZ) $\Delta$ F), pGEM/NP, pGEM/P, 及びpGEM/L(Kato, A. et al., Genes Cells 1, 569-579(1996)), をそれぞれ 12  $\mu$ g, 4  $\mu$ g, 2  $\mu$ g, 4  $\mu$ g/dish およびエンベローププラスミド pGEM/FHN を4  $\mu$ g/dish加え、Opti-MEM(GIBCO) に懸濁し、SuperFect transfection reagent (1  $\mu$ g DNA/5  $\mu$ l のSuperFect, QIAGEN)を入れ、室温で 15 分間放置後、最終的に 3% FBSを含むOpti-MEM 3 ml に入れた DNA-SuperFect 混合物を細胞に添加して培養した。3 時間培養後、細胞を、血清を含まない MEM で 2 回洗浄し、シトシン $\beta$ -D-アラビノフラノシド 40  $\mu$ g/ml (AraC, Sigma) とトリブシン 7.5  $\mu$ g/ml を含むMEMで 24 時間培養した。培養上清を取り除き、血清を含まない MEM培地(40  $\mu$

g/ml AraC, 7.5  $\mu$ g/ml トリブシンを含む) に懸濁された100 mm ペトリ皿 1 枚分の F 発現 LLC-MK2/F7 細胞懸濁液 5 ml を重層した。さらに培養48時間後、これらの細胞と上清を回収し、それぞれ P0-d3 サンプルとした。P0-d3 のペレットを Opti-MEM 培地 に懸濁し ( $2 \times 10^7$  cells/ml)、凍結融解を 3 回繰り返して lipofection reagent DOSPER (Boehringer mannheim) と混合し ( $10^6$  cells/25  $\mu$ l DOSPER) 室温で 15 分間放置した後、F 発現 LLC-MK2/F7 細胞株にトランスフェクション ( $10^6$  cells/well 24-well-plate) し、血清を含まない MEM 培地 (40  $\mu$ g/ml AraC, 7.5  $\mu$ g/ml トリブシンを含む) で培養した。培養後 7 日目に上清を回収し、P1-d7 サンプルとした。さらに上清全量を 12-well-plate に撹いた F 発現 LLC-MK2/F7 細胞株に 37 °C 1 時間感染後、MEM 培地 培地で一回洗浄した後、血清を含まない MEM 培地 (40  $\mu$ g/ml AraC, 7.5  $\mu$ g/ml トリブシンを含む) で培養した。培養後 7 日目に上清を回収し、P2-d7 サンプルとした。さらに上清全量を 6-well-plate に撹いた F 発現 LLC-MK2/F7 細胞株に 37 °C 1 時間感染後、MEM 培地 培地で一回洗浄した後、血清を含まない MEM 培地 (7.5  $\mu$ g/ml トリブシンを含む) で培養した。培養後 7 日目に上清を回収し、P3-d7 サンプルとした。さらに上清全量を 10 cm plate に撹いた F 発現 LLC-MK2/F7 細胞株に 37 °C 1 時間感染後、MEM 培地 培地で一回洗浄した後、血清を含まない MEM 培地 (40  $\mu$ g/ml AraC, 7.5  $\mu$ g/ml トリブシンを含む) で培養した。培養後 7 日目に上清を回収し、P4-d7 サンプルとした。

< LacZ 発現細胞のカウントによる CIU の測定 (LacZ-CIU) >

LLC-MK2 細胞を  $2.5 \times 10^6$  cells/well で 6 well-plate に蒔き、24 時間培養後、血清を含まない MEM 培地 で 1 回洗浄した後、P3-d7 の 1/10 希釈系列を MEM 培地で作製し、37 °C 1 時間感染後、MEM 培地で一回洗浄し、10% 血清を含む MEM 培地 1.5 ml を添加した。37 °C で 3 日培養後、細胞を  $\beta$ -Gal 染色キット (インビトロジェン社) により染色した。3 回の実験の結果を図 4 4 に示す。LacZ 染色陽性細胞数を数えた結果、いずれの場合でも P3-d7 サンプルにおいて  $1 \times 10^6$  CIU/ml のウイルスが得られていることがわかった。

[実施例 15] センダイウイルスにおける極性効果を利用した遺伝子発現量の制御

<SeVゲノムcDNAの構築>

センダイウイルス (SeV) 全長ゲノムcDNA、pSeV(+) (Kato, A. et al., Genes to Cells 1: 569-579, 1996)のcDNAに新たな *Not* I サイトを各遺伝子のスタートシグナルとATG翻訳開始シグナルの間に導入した。導入方法としてはまず、図45(A)のようにpSeV(+)を *Sph* I/*Sal* Iで消化した断片 (2645bp)、*Cla* Iで消化した断片 (3246bp)、及び *Cla* I/*Eco* RIで消化した断片 (5146bp) をそれぞれアガロース電気泳動で分離、該当するバンドを切り出し、QIAEXII Gel Extraction System (QIAGEN社製) で回収・精製した。*Sph* I/*Sal* Iで消化した断片はLITMUS38 (NEW ENGLAND BIOLABS社製)、*Cla* Iで消化した断片と *Cla* I/*Eco* RIで消化した断片はpBluescriptII KS+ (STRATAGENE社製) にライゲーションし、サブクローニングした。続いて *Not* Iサイトの導入にはQuickchange Site-Directed Mutagenesis kit (STRATAGENE社製) を使った。それぞれの導入に用いたプライマーはNP-P間ではセンス鎖: 5'-ccaccgaccacacccagcgccgcgacagccacggcttcgg-3' (配列番号: 19)、アンチセンス鎖: 5'-ccgaagccgtggctgtcgcggccgctgggtgtggtcggtgg-3' (配列番号: 20)、P-M間ではセンス鎖: 5'-gaaatttcacctaagcgccgcaatggcagatatctatag-3' (配列番号: 21)、アンチセンス鎖: 5'-ctatagatatctgccattgcggccgcttaggtgaaatttc-3' (配列番号: 22)、M-F間ではセンス鎖: 5'-gggataaagtccttgcggccgcttggttgcaaaactctcccc-3' (配列番号: 23)、アンチセンス鎖: 5'-gggagagttttgcaaccaagcgccgcaaggactttatccc-3' (配列番号: 24)、F-HN間ではセンス鎖: 5'-ggtcgcgcggtacttttagcgccgcctcaaacaagcacagatcatgg-3' (配列番号: 25)、アンチセンス鎖: 5'-ccatgatctgtgcttgtttgaggcgccgctaaagtaccgcgcgacc-3' (配列番号: 26)、HN-L間ではセンス鎖: 5'-cctgcccattccatgacctagcgccgcttccattcaccctggg-3' (配列番号: 27)、アンチセンス鎖: 5'-cccagggtgaattggaagcgccgctaggtcatggatgggcagg-3' (配列番号: 28) をそれぞれ合成し、



/SEAPとした。

#### <ウイルスの再構築>

LLC-MK2細胞を $2 \times 10^6$  cells/dish で100mmシャーレに蒔き、24時間後培養後、ソラレンとUV処理したT7ポリメラーゼを発現するリコンビナントワクシニアウイルス (PLWUV-VacT7) (Fuerst, T.R. et al., Proc. Natl. Acad. Sci. USA 83: 8122-8126, 1986、Kato, A. et al., Genes Cells 1: 569-579, 1996) に室温でmoi=2で1時間感染させた。細胞を洗浄してからSEAPを組み込んだ各センダイウイルス cDNA、pGEM/NP、pGEM/P、およびpGEM/Lをそれぞれ12 $\mu$ g、4 $\mu$ g、2 $\mu$ g、及び4 $\mu$ g/dishの量比でOptiMEM (GIBCO BRL社製) に懸濁し、110 $\mu$ l のSuperFect transfection reagent (QIAGEN社製) を入れて混合し、室温で15分放置後、最終的に3%FBSを含むOptiMEM 3mlを加え、細胞に添加して3~5時間培養した。培養後、細胞を血清を含まないMEMで2回洗浄し、シトシン $\beta$ -D-アラビノフラノシド (AraC) を含むMEMで72時間培養した。これらの細胞を回収し、ペレットを1mlのPBSで懸濁し、凍結融解を3回繰り返した。これらを10日間孵卵させた鶏卵に100 $\mu$ l接種し、35°Cで3日間孵卵させたのち、尿液を回収した。ワクシニアウイルスフリーにするため、これら回収した尿液をさらに $10^{-5}$  ~  $10^{-7}$  に希釈して鶏卵に再接種し、同様に回収し、分注して-80°Cにストックした。それぞれのウイルスベクター名をSeVNPP/SEAP、SeVPM/SEAP、SeVMF/SEAP、SeVFHN/SEAP、SeVHNL/SEAPおよびSeV18/SEAPとする)。

#### <ブランクアッセイによるタイターの測定>

CV-1細胞を6well プレートに1wellあたり $5 \times 10^5$  cellsずつ蒔き、24時間培養した。PBS洗浄後、BSA/PBS (1% BSA in PBS) で $10^{-3}$ 、 $10^{-4}$ 、 $10^{-5}$ 、 $10^{-6}$ 、 $10^{-7}$  に希釈した組換えSeVを1時間インキュベーションした後、PBSで洗浄、BSA/MEM/アガロース (0.2% BSA+2 $\times$ MEMと等量の2%アガロースを混合したもの) をwellあたり3mlずつ重層し、6日間37°C、0.5%で培養した。培養後、3mlのエタノール/酢酸 (エタノール: 酢酸=1:5) を加え、3時間放置し、アガロースとともに除去した。PBSで

三回洗浄後、100倍希釈したウサギ抗センダイウイルス抗体で室温で1時間インキュベーションした。PBSで三回洗浄後、200倍希釈したAlexa Flour™ 標識ヤギ抗ウサギIg(G+H) (Molecular Probe社) を加えて室温で1時間インキュベーションした。PBSで三回洗浄後、ルミノイメージアナライザーLAS1000 (富士フィルム) で蛍光画像を取り込み、ブランクを測定した。結果を図47に示す。またこれから得られたタイターの結果を表3に示す。

表3 プラークアッセイの結果から測定した各組換えセンダイウイルスのタイターの結果

組み換えウイルス	タイター (pfu/ml)
SeV18/SEAP	3.9X10 <sup>9</sup>
SeVNPP/SEAP	4.7X10 <sup>8</sup>
SeVPM/SEAP	3.8X10 <sup>9</sup>
SeVMF/SEAP	1.5X10 <sup>10</sup>
SeVFHN/SEAP	7.0X10 <sup>9</sup>
SeVHNL/SEAP	7.1X10 <sup>9</sup>

#### <レポーター遺伝子発現の比較>

LLC-MK2細胞を6well プレートに1wellあたり $1\sim 5\times 10^5$  cellsずつ蒔き、24時間培養した後、各ウイルスベクターをmoi=2感染させ、24時間後培養上清を100 $\mu$ l 回収し、SEAPアッセイを行った。アッセイはReporter Assay Kit -SEAP- (東洋紡) で行い、ルミノイメージアナライザーLAS1000 (富士フィルム) で測定した。測定値はSeV18+/SEAPの値を100としてそれぞれ相対値として表した。その結果、図48に示したいずれの位置にSEAP遺伝子を挿入した場合でもSEAP活性が検出された。SEAP活性はゲノムの下流に位置するに従って下がり、すなわち発現量が下がっていることがわかった。また、NP遺伝子とP遺伝子の間にSEAP遺伝子を挿入した場

合には、NP遺伝子の上流にSEAP遺伝子を挿入したベクターと、P遺伝子とM遺伝子の間にSEAP遺伝子を挿入したベクターの中間の発現量が検出された。

【実施例 16】 ダブル欠失 $\Delta$ F-HN細胞重層法による欠失SeV増幅効率の向上

現在用いているSeVウイルスの再構築法では、T7 RNAポリメラーゼを発現する組換えワクシニア(vTF7-3)を用いるため、ワクシニアの細胞傷害性により、感染細胞が一部分死滅しており、再構築を行った一部の細胞でウイルスが増幅することができても、さらに多くの細胞で効率よく、持続的に増幅できるようにすることが好ましい。しかし、パラミクソウイルスでは同型ウイルスのFとHN蛋白が細胞表面に共に存在すると細胞融合を引き起こし、シンシチウムが形成されることが知られている (Lamb and Kolakofsky, 1996, Fields virology, p1189)。それ故FHN共発現細胞の継代が困難であった。そこで、これらの再構築された細胞に新たに欠失蛋白 (FおよびHN) を発現するヘルパー細胞を重層することにより欠失ウイルスの回収効率を向上することができると考えた。FHN発現誘導時間が異なる細胞を重層することを検討することによりFHN共欠失ウイルス回収効率を大きく向上した。

10cm細胞培養皿に100%コンフレントになったLLC-MK2細胞 ( $1 \times 10^7$ /dish)をPLWUV-処理ワクシニアをmoi=2で室温において感染1時間後、d2EGFPを搭載するFHN欠失cDNA (pSeV18'/ $\Delta$ FHN-d2GFP (実施例 8)), pGEM/NP, pGEM/P, pGEM/L, pGEM/FHNをそれぞれ、12 $\mu$ g/10cm dish, 4 $\mu$ g/10cm dish, 2 $\mu$ g/10cm dish, 4 $\mu$ g/10cm dish, 4 $\mu$ g/10cm dishの量比で混合し (final vol, 3ml/10cm dish)、遺伝子導入試薬 SuperFect(QIAGEN)を用いて、前述したF欠失ウイルスの再構築と同様な方法でLLC-MK2細胞に遺伝子導入した。遺伝子導入3時間後細胞を無血清培地で3回洗浄し、低速遠心(1000rpm/2min)で剥がれた細胞を回収し、シトシン $\beta$ -D-アラビノフラノシド (AraC) 40 $\mu$ g/ml, SIGMA), トリプシン(7.5 $\mu$ g/ml, GIBCO)を含むの無血清MEM培地に懸濁し、細胞に加え、一晚培養した。別途に用意した10cmシャーレで100%コンフレントになったFHN共発現細胞をアデノウイルスAxCANCre

- 74 -

をMOI=10で発現誘導後、4時間、6時間、8時間、2日目、3日目の細胞をそれぞれ5ml PBS(-)で一回洗浄し、cell dissociation solution (SIGMA)により細胞を剥がし、低速遠心(1000rpm/2min)で細胞を集め、AraC(40 $\mu$ g/ml, SIGMA)、トリプシン(7.5 $\mu$ g/ml, GIBCO)を含むの無血清MEM培地に懸濁し、FHN共欠失ウイルスの再構築した細胞(P0)に加え一晚培養した。細胞重層後2日目で蛍光顕微鏡で細胞を観察し、細胞におけるGFPの発現でウイルスの広がりを確認した。その結果、図49に示した。細胞重層しない従来の場合(左)に比べ、細胞を重層した場合(右)は重層された細胞の方がGFP発現細胞が顕著に多く認められた。これらの細胞を回収し、10<sup>7</sup>細胞/mlのOpti-MEM培地(Gibco)に懸濁し、3回凍結融解したライセートを調製し、発現誘導して2日後のFHN共発現細胞に10<sup>6</sup> cells/100 $\mu$ l/well感染し、AraC(40 $\mu$ g/ml, SIGMA)、トリプシン(7.5 $\mu$ g/ml, GIBCO)を含むの無血清MEM培地で、37°C 5%CO<sub>2</sub> インキュベーターで2日間培養したP1細胞培養上清のウイルス力価をCIU-GFPで測定した(表4)。その結果、FHN発現誘導後4時間ではウイルスの増幅効果が認められず、誘導6時間以後の細胞重層による増幅効果が顕著に認められた。特に、P1細胞上清中に放出されたウイルスは6時間後の細胞重層する方が細胞重層しない方に比べ約10倍に上った。

表4 ダブル欠失 $\Delta$ F-HN細胞重層法による欠失SeV増幅

GFP -CIU		x10 <sup>3</sup> /ml				
		FHNcell+ad/cre				
FHN cell-		4h	6h	8h	2d	3d
8-10		6-9	80-100	70-100	60-100	20-50

[実施例17] シュードタイプセンダイウイルスがF欠失型ゲノムを有することの確認



VSV-G遺伝子発現で増殖した上記ウィルスがF欠失型であることを、感染細胞抽出液のタンパク質のウェスタン解析により調べた。その結果、センダイウィルス由来のタンパク質は検出されるものの、Fタンパク質は検出されなかったことから、F欠失型であることが確認された（図50）。

【実施例18】 FおよびHN遺伝子を欠失したゲノムを有するシュードタイプセンダイウィルスの抗VSV抗体による感染性の影響

VSV-G発現株を用いて得られたFおよびHN遺伝子を欠失したゲノムを有するシュードタイプセンダイウィルスが、外被にVSV-Gタンパク質を有するかに関して、抗VSV抗体を用いて感染性が影響されるかどうかの中和活性を調べた。ウィルス液と抗体を混合し、室温で30分静置後、VSV-Gを誘導発現していないLLCG-L1細胞に感染し、4日目の遺伝子導入能をGFP発現細胞の有無で調べた。その結果、FおよびHN遺伝子を欠失したゲノムを有するシュードタイプセンダイウィルス（図中VSV-G）は、抗VSV抗体で感染性の完全な抑制が認められたが、本来の外被を有するセンダイウィルス（図中F,HN）では抑制が認められなかった（図51）。このことから、本実施例で得られたウィルスが、外被にVSV-Gタンパク質を有するシュードタイプのセンダイウィルスであり、抗体によりその感染性が特異的に抑えられることが明らかとなった。

【実施例19】 F遺伝子とFおよびHN遺伝子を欠失したゲノムを有するシュードタイプセンダイウィルスの密度勾配超遠心法を用いた精製

ウィルス感染細胞の培養上清を用いて、ショ糖密度勾配遠心を行い、F遺伝子とFおよびHN遺伝子を欠失したゲノムを有するシュードタイプセンダイウィルスの分画精製を行った。20～60%のグラジェントを形成させたショ糖溶液にウィルス液を上層させ、SW41ローター（Beckman）で29000rpm、15～16時間超遠心を行った。超遠心後チューブの底に穴を開け、フラクションコレクターで300 $\mu$ lずつ分画した。各画分について、F遺伝子あるいはFおよびHN遺伝子を欠失したゲノムを有し、外被にVSV-Gタンパク質を有するシュードタイプのセンダイウィルスであること

をウェスタン解析により調べた。ウェスタン解析は、上記に記載の方法により行った。その結果、F欠失型のシュードタイプセンダイウィルスでは、センダイウィルス由来のタンパク質およびHNタンパク質、VSV-Gタンパク質は同フラクションに検出されるものの、Fタンパク質は検出されなかったことから、F欠失型シュードタイプのセンダイウィルスであることが確認された。一方、FおよびHN欠失型のシュードタイプセンダイウィルスでは、センダイウィルス由来のタンパク質、VSV-Gタンパク質は同フラクションに検出されるものの、FおよびHNタンパク質は検出されなかったことから、FおよびHN欠失型のシュードタイプのセンダイウィルスであることが確認された（図52）。

〔実施例20〕 F遺伝子とFおよびHN遺伝子を欠失したゲノムを有するシュードタイプセンダイウィルスによる赤血球凝集反応の回避

F遺伝子、またはF、HN遺伝子を欠失したゲノムを有するシュードタイプセンダイウィルス、あるいは本来の外被を有するセンダイウィルスをLLC-MK2細胞に感染させ、3日目に1%トリ赤血球浮遊液を加え4℃で30分静置後、GFPを発現した感染細胞表面を観察した。その結果、F遺伝子を欠失したゲノムを有するウィルス（SeV/ $\Delta$ F、およびVSV-Gでシュード化した SeV/ $\Delta$ F(VSV-G)）シュードタイプセンダイウィルスは本来の外被を有するセンダイウィルスと共に、感染細胞の表面に凝集反応が起きているのが確認された。一方、FおよびHN遺伝子を欠失したゲノムを有するシュードタイプセンダイウィルス（SeV/ $\Delta$ F-HN(VSV-G)）では、感染細胞上に凝集は全く起きていないことが明らかとなった（図53）。

〔実施例21〕 F遺伝子を欠失したゲノムを有するVSV-Gシュードタイプセンダイウィルスによる培養細胞への感染特異性

培養細胞へのF遺伝子を欠失したゲノムを有するVSV-Gシュードタイプセンダイウィルスの感染効率は、細胞に感染後3日目の生細胞に発現したGFP量をFlow cytometryを用いて測定した。F遺伝子を欠失したゲノムを有するシュードタイプセンダイウィルスと本来の外被を有するセンダイウィルスで、ほぼ同じ感染効率

を示すLLC-MK2細胞をコントロールとして比較を行った。その結果、ヒト卵巣ガン細胞HRAでの感染効率は、LLC-MK2細胞とほとんど差異はなかったが、T細胞系のJurkat細胞では、コントロールと比較して2倍程度のF遺伝子を欠失したゲノムを有するVSV-Gシュードタイプセンダイウィルスの感染効率の上昇が観察された(図54)。

[実施例22] NGF発現を搭載したF欠失型センダイウィルスベクターの作製  
<NGF/SeV/ $\Delta$ Fの再構成>

NGF/SeV/ $\Delta$ Fの再構成は上記「エンベローププラスミド+F発現細胞重層法」に従って行った。また、タイターの測定は、抗SeVポリクローナル抗体を用いた方法に従って行った。

<NGF/SeV/ $\Delta$ Fのウィルスゲノム確認(RT-PCR)>

NGF/SeV/ $\Delta$ Fウィルスゲノム(図55上)を確認するため、LLC-MK2/F7細胞から回収された培養上清を遠心した後、QIAamp Viral RNA mini kit (QIAGEN) でそのプロトコールに従ってRNA抽出を行った。このRNAをSUPERScript™ ONE-STEP™ RT-PCR SYSTEM (GIBCO BRL) によりRT-PCRのテンプレート合成およびPCRを行った。対照群は、付加型SeV cDNA (pSeV18' b(+)) (Hasan, M. K. et al., J. General Virology 78: 2813-2820, 1997) を用いた。PCRプライマーはNGF-NとNGF-Cを用いて行った。NGF-Nについては、フォワード: ACTTGCGGCCGCCAAAGTTCAGTAATGTCCA TGTGTCTTACTACTCTG (配列番号: 33)、NGF-Cについては、リバーズ: ATCCGCGGC CGCGATGAACCTTACCCCTAAGTTTTCTTACTACGGTCAGCCTCTTCTGTAGCCTTCCTGC (配列番号: 34) を使用した。その結果、NGF-NとNGF-Cとをプライマーに用いた場合は、RT条件下でNGF/SeV/ $\Delta$ FはNGFに特異的なバンドが検出された。対照群にはバンドは検出されなかった(図55下)。

[実施例23] NGF遺伝子を搭載したF欠失型SeVの細胞感染後に発現するNGF蛋白の定量とin vitro活性測定

感染及びNGF蛋白の発現は、直径10cm或いは直径6cmプレートにほぼconfluent

に増殖させた LLC-MK2/F 或いは LLC-MK2 細胞を用いて行った。NGF/SeV/  $\Delta$  F, NGF/SeV/  $\Delta$  F-GFP は LLC-MK2/F 細胞に、NGF/SeV 及び GFP/SeV は LLC-MK2 細胞に m.o.i. 0.01 で感染させ、7.5  $\mu$ g/mL の Trypsin (GIBCO) を含み血清を含まない MEM 培地で3日間培養した。3日後ほぼ100%の細胞が感染した後に、Trypsin 及び血清を共に含まない MEM 培地に交換し更に3日間培養した。それぞれの培養上清を回収し、48,000  $\times$  g にて60分遠心後、上清について NGF 蛋白の定量及び in vitro 活性測定を行った。本実施例では、F 欠失型 SeV (NGF/SeV/  $\Delta$  F, NGF/SeV/  $\Delta$  F-GFP) (図 5 5 参照) を LLC-MK2/F 細胞に感染させているが、高 m.o.i. (例えば1或いは3) を感染すれば、即ちはじめから100%近い細胞に感染すれば、当然のことながら F 非発現細胞でも同様の結果を示す実験を行うことができる。

NGF 蛋白の定量は ELISA Kit である NGF Emax Immuno Assay System (Promega) を利用した。プロトコールは添付文書の指示に従った。NGF/SeV/  $\Delta$  F, NGF/SeV/  $\Delta$  F-GFP 及び NGF/SeV の感染細胞培養上清中にはそれぞれ 32.4  $\mu$ g/mL, 37.4  $\mu$ g/mL 及び 10.5  $\mu$ g/mL の NGF 蛋白の存在が確認された。NGF/SeV/  $\Delta$  F, NGF/SeV/  $\Delta$  F-GFP の感染細胞培養上清中には、高濃度の NGF 蛋白が存在し NGF/SeV の感染細胞培養上清中の NGF 蛋白量と同程度であり、F 欠失型 SeV によっても十分量の NGF の発現があることが確認された。

NGF 蛋白の in vitro 活性測定は、ニワトリの感覚神経である後根神経節の初代神経細胞分散培養系での生存維持活性を指標に行った (Nerve Growth Factors (Wiley, New York), pp.95-109 (1989))。胎生10日齢のニワトリ胚より後根神経節を取り出し、0.25% Trypsin (GIBCO) で 37°C 20分処理後分散した。100 units/mL の penicillin (Gibco), 100 units/mL の streptomycin (Gibco), 250 ng/mL の amphotericin B (Gibco), 20  $\mu$  M の 2-deoxyuridine (Nakarai), 20  $\mu$  M の 5-fluorodeoxyuridine (Nakarai), 2 mM L-glutamine (Sigma) 及び 5% の血清を含む高グルコースの D-MEM 培地を使用し、96-well プレートに 1 well あたり約 5000 個の細胞密度で培養を開始した。プレートは polylysine コートした 96-well プレート (Iwaki)

を更にlaminin (Sigma) でコートして準備した。培養開始時にコントロールであるNGF蛋白或いは先に調製したSeV感染後の培養上清を添加した。3日後、顕微鏡下で細胞を観察すると共に、Alamer blue (CosmoBio) を添加しミトコンドリアによる還元活性を指標として (530nmで励起した590nmの蛍光強度を測定) 生細胞の定量を行った。コントロール (NGF添加無し) 及びSeV/付加型-GFP (GFP/SeV) の感染細胞培養上清の添加 (1/1000希釈) では同程度の生細胞を示す蛍光強度であったが、NGF/SeV/ $\Delta$ F, NGF/SeV/ $\Delta$ F-GFP及びNGF/SeVの感染細胞培養上清を添加 (1/1000希釈) することにより、顕著な蛍光強度の上昇が見られ生細胞数が多く生存維持活性を有していると判断された (図5 6)。そして、その値はELISAにより求めたNGF蛋白量の添加に匹敵する効果であった。同様のことが顕微鏡下で視覚的にも観察され、NGF/SeV/ $\Delta$ F, NGF/SeV/ $\Delta$ F-GFP及びNGF/SeVの感染細胞培養上清を添加することにより、生細胞数の増加と顕著な突起伸展が観察された (図5 7)。即ち、NGF搭載F欠失型SeVの感染によって発現されるNGFは活性型として発現していると確認された。

#### 【実施例2 4】 F発現細胞の詳細な解析

##### 1) Adeno-Creのmoiと誘導時間

異なるAdeno-Creのmoiを使ってLLC-MK2/Fに感染させF蛋白の発現を誘導した後、蛋白の発現量と細胞の形態変化を調べた。

moi=1の場合に比べmoi=10の場合発現量が若干高かったが (図5 8)、誘導後6h、12h、24h、48h後の発現量を調べたところ、いずれも誘導後48時間目にF蛋白の発現量が高いことが分かった。

また、moi=1、3、10、30、100で細胞に感染して細胞の形態変化を経時的に観察したが、moi=10までに細胞間に顕著の差が認められなかったが、moi=30以上になると細胞傷害性が観察された (図5 9)。

##### 2) 継代数

LLC-MK2/Fに対してAdeno-Creを使ってF蛋白の発現を誘導してから7代まで継代し

、細胞のFの発現量と細胞の形態を顕微鏡観察で調べた。一方、F蛋白の発現を誘導してから20代まで継代した細胞内F蛋白の存在状態をレーザー顕微鏡を用いて調べた。

レーザー顕微鏡観察においては、チャーンバーガラスにF蛋白の発現を誘導したLLC-MK2/F細胞を入れ、一晚培養した後、培地を取り除きPBSで一回洗浄してから、3.7%のFormalin-PBSで5分間固定した。その後、PBSで細胞を一回洗浄した後、0.1% Triton X100-PBSで5分間処理して、抗F蛋白モノクローナル抗体( $\gamma$ -236)(100倍希釈)とFITC標識山羊抗ウサギIgG抗体(200倍)の順で細胞を処理して、最後にPBSで洗浄してレーザー顕微鏡をもって観察した。

その結果、7代目まで継代した細胞のF蛋白の発現量に差はなかった(図60)。形態的にも、そしてSeVの感染性と生産性にも顕著な差が観察されなかった。一方、20代目まで継代した細胞を免疫抗体法で細胞内のF蛋白の存在状況を調べたところ、15代まで大きな差がなかったが、それ以上継代した細胞内にF蛋白の局在化傾向が観察された(図61)。

以上の結果から、F欠失型SeVの生産には継代後15代目までの細胞が望ましいと判断される。

#### 【実施例25】 GFP-CIUと抗SeV-CIUとの相関関係

2種類の方法によるCIU (Cell-Infected Unit) の測定結果を相関関係を調べた。LLC-MK2細胞を $2 \times 10^5$  cells/dishで12well-plateに蒔き、24時間培養後、血清を含まないMEM培地で1回洗浄した後、SeV/ $\Delta$ F-GFPを100  $\mu$ l/wellで感染した。15分後、血清を含まないMEM培地を1ml/well加え、さらに24時間培養した。培養後、PBS(-)で3回洗浄した後、細胞を乾燥させ(約10分～15分室温放置)、細胞を固定するため、アセトンを1 ml/well加え直ちにに取り除き、再び乾燥させた(約10分～15分室温放置)。PBS(-)で100倍希釈したウサギより調製された抗SeVポリクローナル抗体(DN-1)を300  $\mu$ l/well加え、37°Cで45分間インキュベートした後、PBS(-)で3回洗浄し、PBS(-)で200倍希釈した抗ウサギIgG(H+D)蛍光標識二次抗体 (Alex<sup>TM</sup>

568: Molecular Probes社製)を300 $\mu$ l/well加え、37°Cで45分間インキュベートした。PBS(-)で3回洗浄した後、蛍光顕微鏡下 (Emission: 560nm, Absorption: 645nm フィルター:ライカ社製) で蛍光を発する細胞を観察した。

対照として SeV/ $\Delta$ F-GFP を100 $\mu$ l/wellで感染し15分後、血清を含まないMEMを1 ml/well加え、さらに24時間培養後、以後の操作を行わずに細胞を蛍光顕微鏡下 (Emission: 360nm, Absorption: 470nm フィルター:ライカ社製) でGFP発現細胞を観察した。

両者の蛍光強度を定量化して関係性を評価したところ、良好な相関を示した (図62)。

#### [実施例26] マルチクローニングサイトの作製

マルチクローニングサイトをSeVベクターに付加させた。方法は以下の二種類。

- 1) センダイウイルス (SeV) 全長ゲノムcDNA、pSeV18<sup>+</sup> のcDNAのゲノム中のいくつかの制限酵素サイトを壊し、つぶした制限酵素サイトを含む新たな制限酵素サイトを各遺伝子のスタートシグナルとATG翻訳開始シグナルの間に導入した。
- 2) すでに構築したSeVベクターcDNAにマルチクローニングサイト配列と転写開始シグナル-介在配列-終結シグナルを付加させてNotIサイトへ組み込む。

1) の場合、導入方法としてはまず、pSeV18<sup>+</sup> をEag Iで消化した断片 (2644bp)、Cla Iで消化した断片 (3246bp)、ClaI/Eco RIで消化した断片 (5146bp)、及びEco RIで消化した断片 (5010bp) をそれぞれアガロース電気泳動で分離、該当するバンドを切り出し、QIAEXII Gel Extraction System (QIAGEN社製) で回収・精製した。Eag Iで消化した断片はLITMUS38 (NEW ENGLAND BIOLABS社製)、Cla Iで消化した断片、ClaI/Eco RIで消化した断片、及びEco RIで消化した断片はpBluescriptII KS<sup>+</sup> (STRATAGENE社製) にライゲーションし、サブクローニングした。続いて制限酵素サイトの破壊、導入にはQuickchange Site-Directed Mutagenesis kit (STRATAGENE社製) を使った。

制限酵素サイトの破壊にはSal I: (センス鎖) 5'-ggagaagtctcaacaccgtccaccc

aagataatcgatcag-3' (配列番号: 35)、(アンチセンス鎖) 5'-ctgatcgattatcttgggtggacgggtgttgagacttctcc-3' (配列番号: 36)、Nhe I: (センス鎖) 5'-gtat atgtgttcagttgagcttgctgtcgggtctaaggc-3' (配列番号: 37)、(アンチセンス鎖) 5'-gccttagaccgacagcaagctcaactgaacacatatac-3' (配列番号: 38)、Xho I: (センス鎖) 5'-caatgaactctctagagaggctggagtcactaaagagttacctgg-3' (配列番号: 39)、(アンチセンス鎖) 5'-ccaggtaactcttttagtgactccagcctctctagagagttcattg-3' (配列番号: 40)、また制限酵素導入にはNP-P間: (センス鎖) 5'-gtgaaagttcatccaccgatcggctcactcgaggccacacccaacccaccg-3' (配列番号: 41)、(アンチセンス鎖) 5'-cggtggggttggtgtggcctcgagtgagccgatcggatgaactttcac-3' (配列番号: 42)、P-M間: (センス鎖) 5'-cttagggtgaaagaaatttcagctagcacggcgcaatggcagatc-3' (配列番号: 43)、(アンチセンス鎖) 5'-gatatctgccattgcgccgtgctagctgaaatttctttcaccctaag-3' (配列番号: 44)、M-F間: (センス鎖) 5'-cttagggataaagtccttgtgcgcgcttggttgcaaaactctcccc-3' (配列番号: 45)、(アンチセンス鎖) 5'-ggggagagttttgcaaccaagcgcgcacaaaggactttatccctaag-3' (配列番号: 46)、F-HN間: (センス鎖) 5'-ggtcgcgcggtacttttagtcgacacctcaacaagcagatcatgg-3' (配列番号: 47)、(アンチセンス鎖) 5'-ccatgatctgtgcttgtttgaggtgtcgactaaagtaccgcgcgacc-3' (配列番号: 48)、HN-L間: (センス鎖) 5'-ccagggtgaatgggaaggcgccaggtcatggatggcaggagtcc-3' (配列番号: 49)、(アンチセンス鎖) 5'-ggactcctgcccattccatgacctggccggcccttccattcacctggg-3' (配列番号: 50) をそれぞれ合成し反応に用いた。導入後、それぞれの断片を上記同様に回収・精製し、cDNAをアセンブリした。

2) の場合、(センス鎖) 5'-ggccgcttaattaacggtttaaacgcgcgccaacagtggtgataagaaaaacttagggtgaaagttcatcac-3' (配列番号: 51)、(アンチセンス鎖) 5'-ggc cgtgatgaactttcacctaagtttttcttatcaacactgttggcgcgctttaaacggttaattaagc-3' (配列番号: 52) を合成し、それぞれの合成DNAをリン酸化し、85°C 2分、65°C 15分、37°C 15分、室温 15分でアニーリングさせ、SeV cDNAへ組み込む。ある



- 83 -

いはpUC18またはpBluescriptII等のマルチクローニングサイトを終結シグナル-  
介在配列-開始シグナル含むプライマーでPCRしてサブクローニングし、これをSe  
V cDNAへ組み込む。できたcDNAでのウイルス再構成は上記の通り行う。

#### 産業上の利用の可能性

本発明により、エンベロープ遺伝子を欠損したパラミクソ科ウイルスベクター  
が提供された。本発明は、マイナス鎖RNAウイルスを基本骨格とした実用化可能な  
新しいエンベロープ遺伝子欠損型ベクターシステムを初めて確立するものである  
。ヘルパー細胞を用いたF遺伝子欠損、FHN遺伝子欠損ゲノムcDNAからの感染性欠  
損ウイルス粒子の回収の成功は、センダイウイルスの優れた特徴を生かした新規  
な遺伝子治療用ベクターの研究開発に道を開いた。本発明の欠損型センダイウ  
イルスベクターは遺伝子導入効率も広範な細胞種に対して極めて高く外来遺伝子を  
驚異的に発現する能力を持っている。さらに、感染細胞で持続的に発現し、2次  
的な感染性ウイルス粒子を放出しないことから、ウイルスの伝播性を完全に無く  
した安全性の高いベクターである。

## 請求の範囲

1. (a) パラミクソ科ウイルスの少なくとも一つのエンベロープタンパク質を発現しないように改変されたパラミクソウイルスに由来する(一)鎖一本鎖RNA、および(b)該(一)鎖一本鎖RNAと結合するタンパク質、からなる複合体を含むパラミクソ科ウイルスベクター。
2. (一)鎖一本鎖RNAがNPタンパク質、Pタンパク質、およびLタンパク質を発現し、Fタンパク質および/またはHNタンパク質を発現しないように改変されている、請求項1に記載のベクター。
3. (一)鎖一本鎖RNAから発現しないように改変されたエンベロープタンパク質の少なくとも一つを含む、請求項1または2に記載のベクター。
4. VSV-Gタンパク質を含む、請求項1から3のいずれかに記載のベクター。
5. (一)鎖一本鎖RNAがセンダイウイルスに由来する、請求項1から4のいずれかに記載のベクター。
6. (一)鎖一本鎖RNAがさらに外来遺伝子をコードしている、請求項1から5のいずれかに記載のベクター。
7. 請求項1から6のいずれかに記載のベクターに含まれる(一)鎖一本鎖RNAまたはその相補鎖をコードするDNA。
8. 請求項1から6のいずれかに記載のベクターの製造方法であって、
  - (a) パラミクソ科ウイルスの少なくとも一つのエンベロープタンパク質を発現しないように改変されたパラミクソウイルスに由来する(一)鎖一本鎖RNAまたはその相補鎖をコードするベクターDNAを、エンベロープタンパク質を発現する細胞に導入して発現させる工程、および
  - (b) 該細胞を培養し、その培養上清からウイルス粒子を回収する工程、を含む方法。
9. 請求項1から6のいずれかに記載のベクターの製造方法であって、

(a) パラミクソ科ウイルスの少なくとも一つのエンベロープタンパク質を発現しないように改変されたパラミクソウイルスに由来する(－)鎖一本鎖RNA、および該(－)鎖一本鎖RNAと結合するタンパク質、からなる複合体を、エンベロープタンパク質を発現する細胞に導入する工程、および

(b) 該細胞を培養し、その培養上清からウイルス粒子を回収する工程、を含む方法。

10. 工程(b)における細胞の培養が、エンベロープタンパク質を発現する細胞との共培養である、請求項8または9に記載の方法。

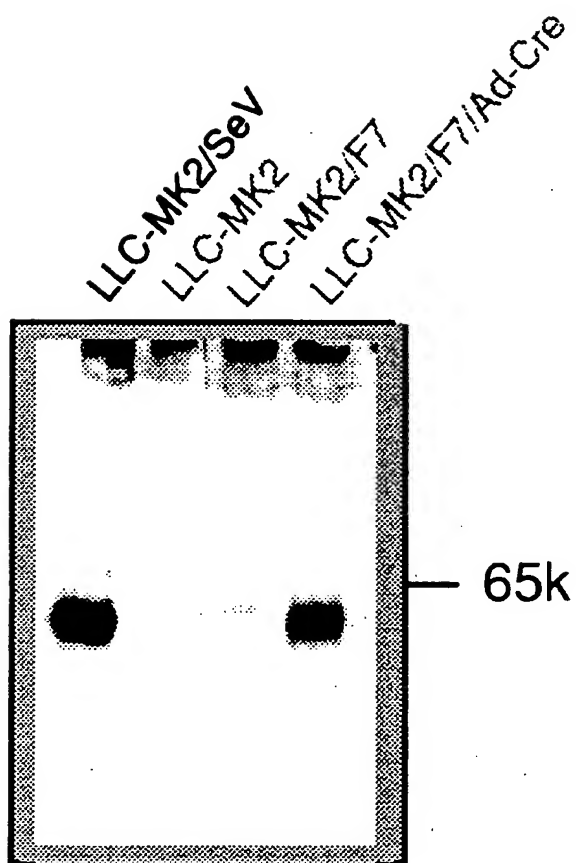
11. 工程(b)における細胞の培養において、該細胞に、エンベロープタンパク質を発現する細胞を重層して培養を行う、請求項8または9に記載の方法。

12. 細胞が発現するエンベロープタンパク質の少なくとも1つが、前記(－)鎖一本鎖RNAから発現しないように改変されたエンベロープタンパク質の少なくとも1つと同一である、請求項8から11のいずれかに記載の方法。

13. 細胞が発現するエンベロープタンパク質の少なくとも1つがVSV-Gタンパク質である、請求項8から12のいずれかに記載の方法。

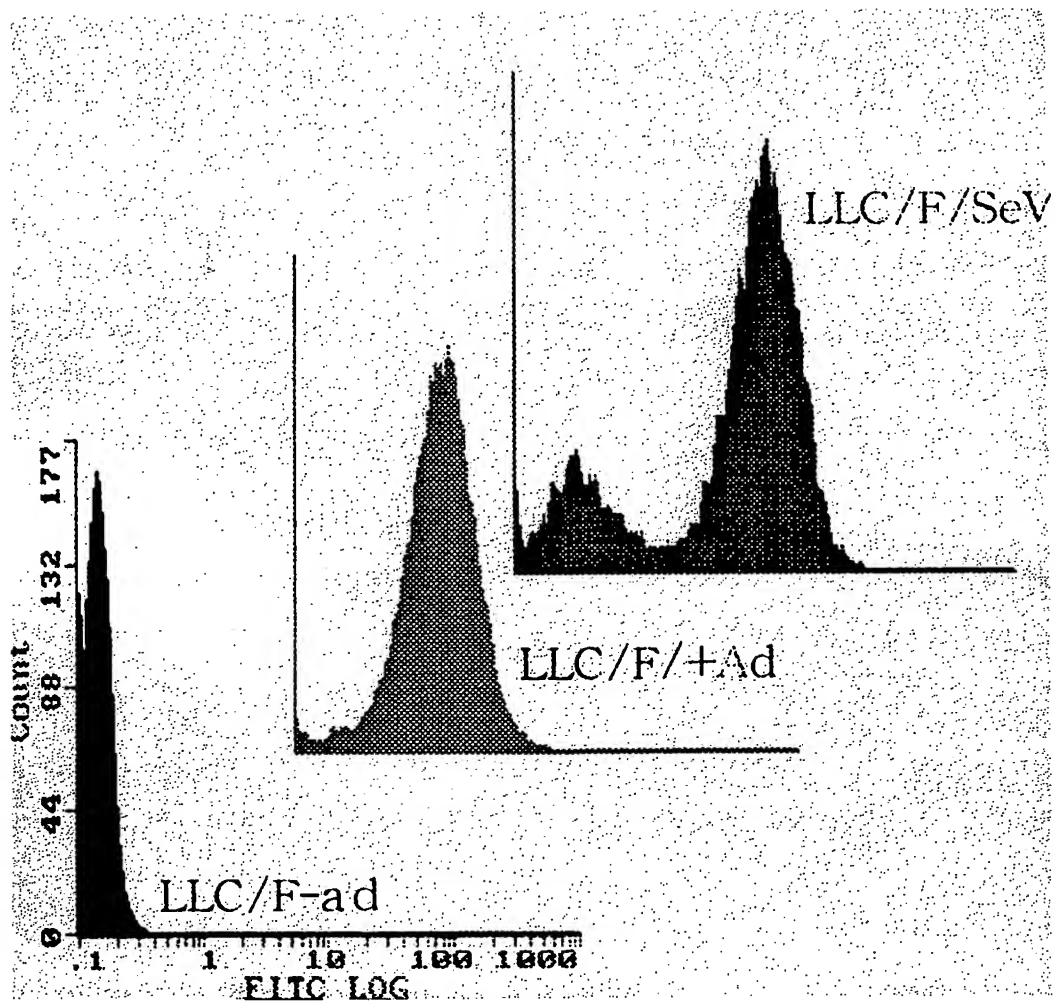
1 / 6 2

☒ 1



2 / 6 2

2



3 / 6 2

図 3



1 : SeV virions

2 : LLCMK2

3 : LLC/F7-trypsin

4 : LLC/F7+trypsin

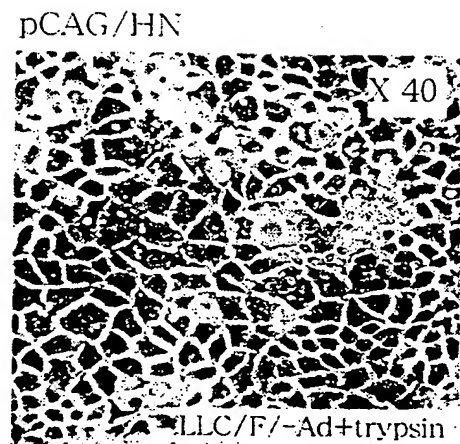
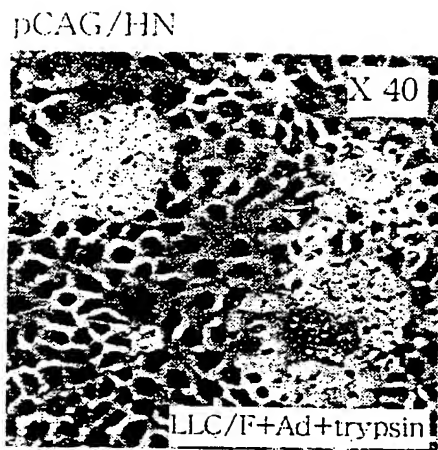
← F 0

← F 1

← F 2

4 / 6 2

図 4



5 / 6 2

図 5

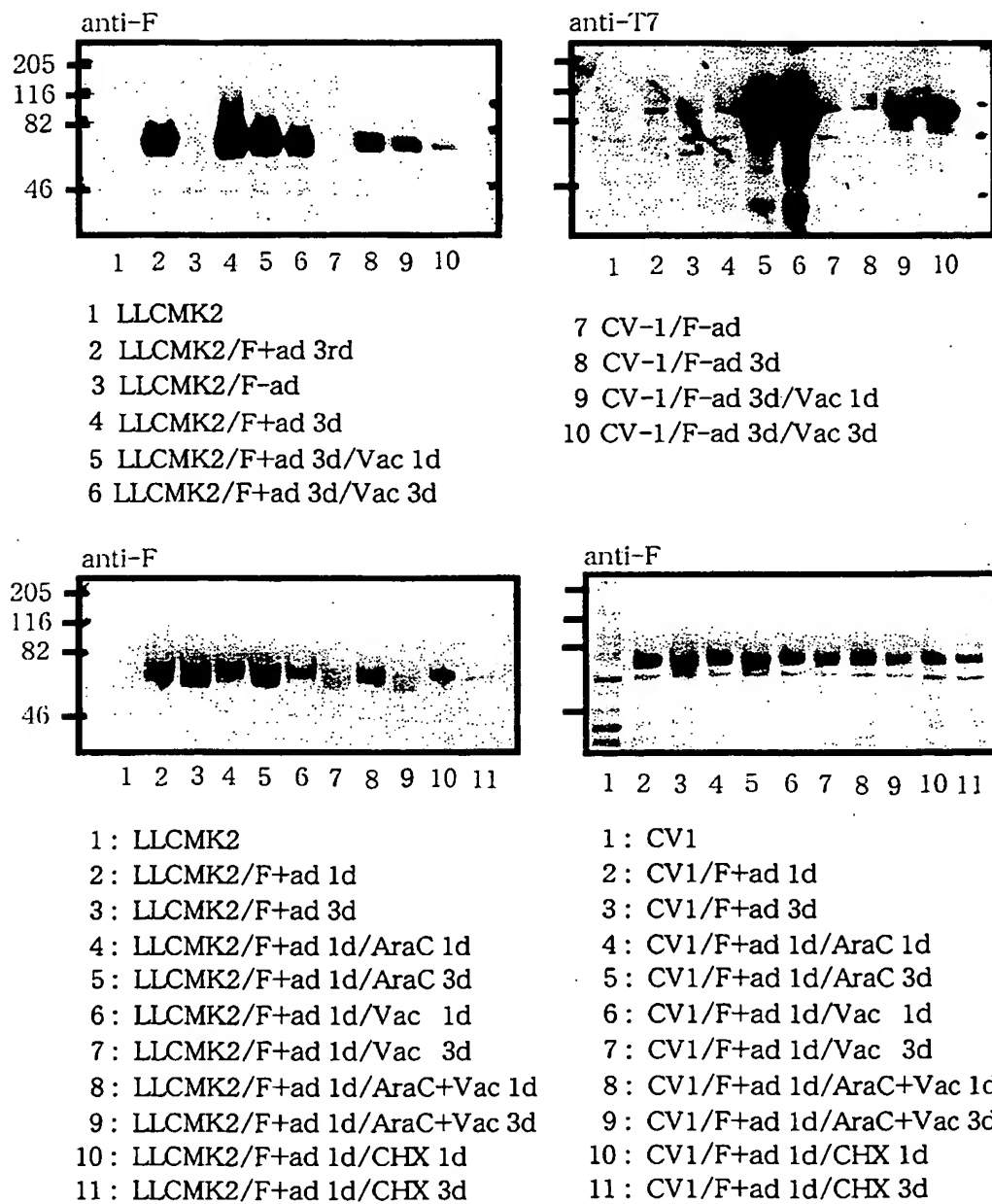
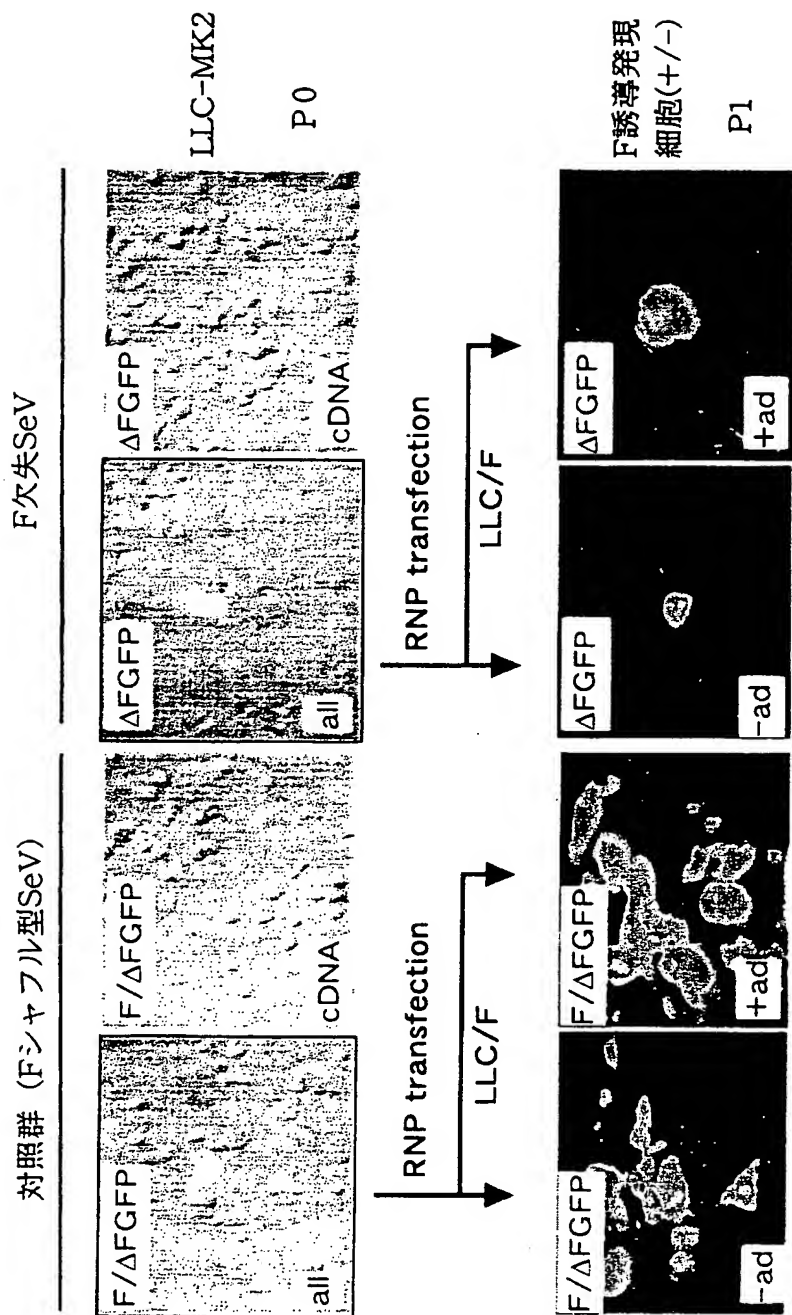




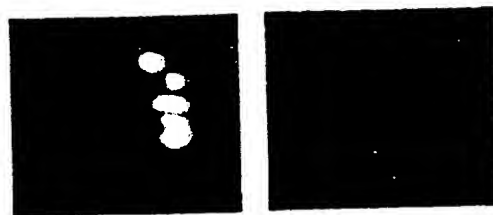
図 6



7 / 6 2

☒ 7

LLCMK2/F  
P1/24 well



Sup

RNP

RNP/o

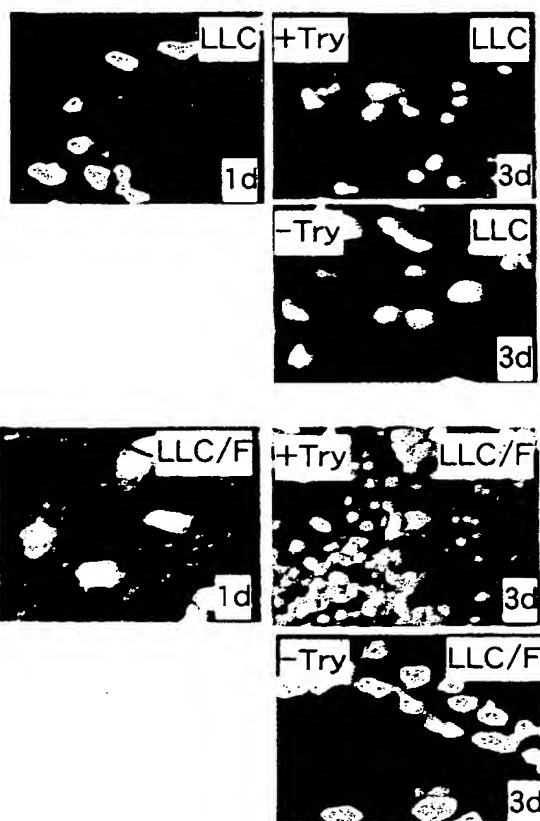
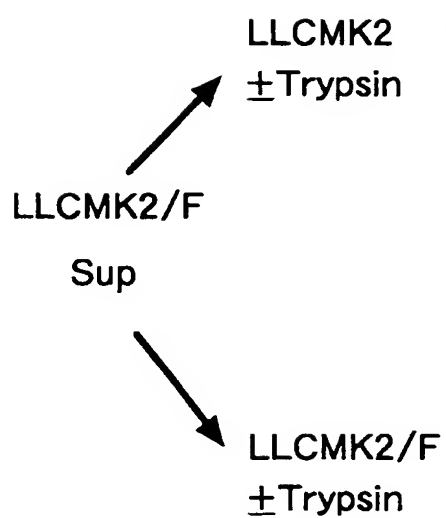
RNP/t

LLCMK2/F  
P2/12 well



8 / 6 2

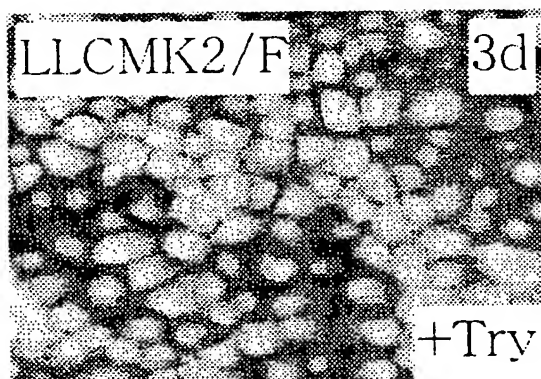
図 8



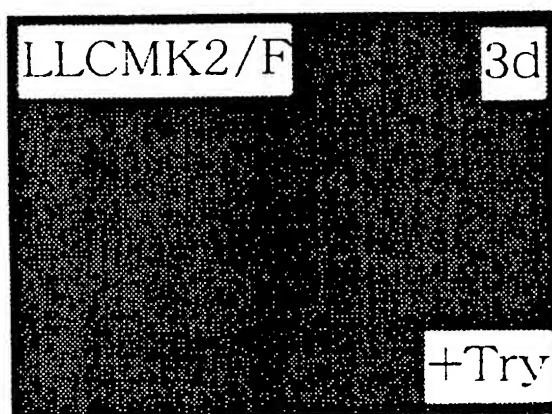
9/62

図9

LLCMK2/F supによる感染

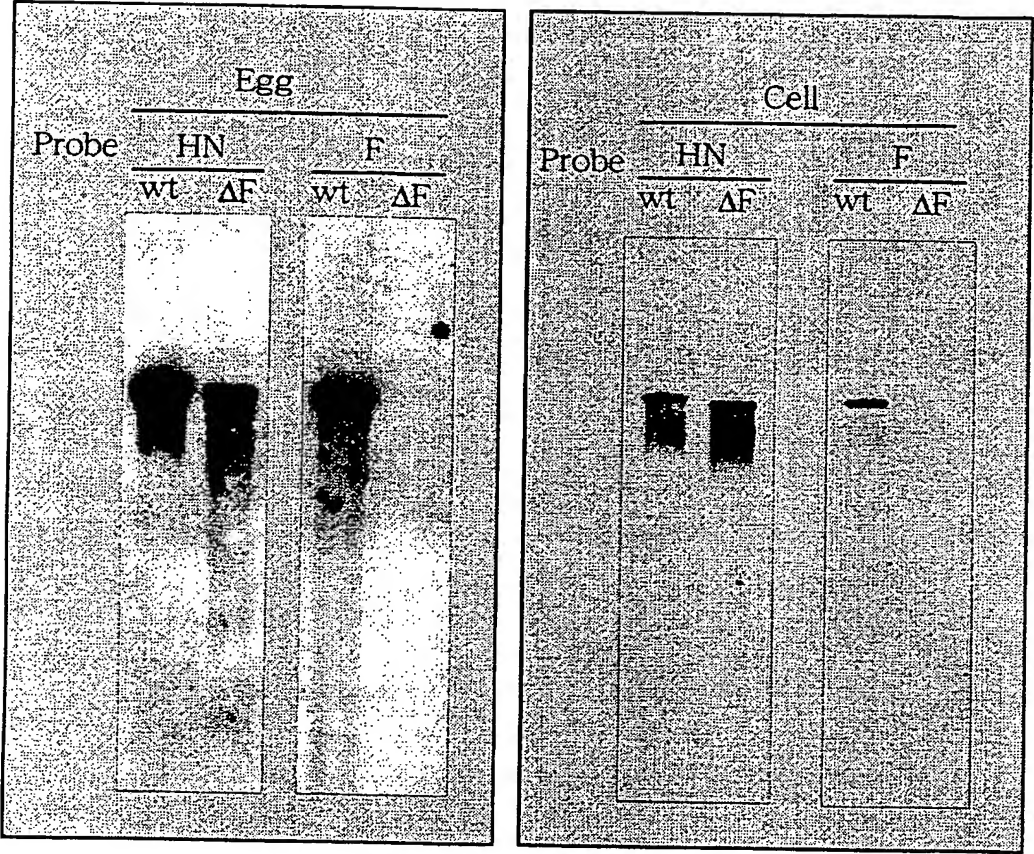


LLCMK2 supによる感染



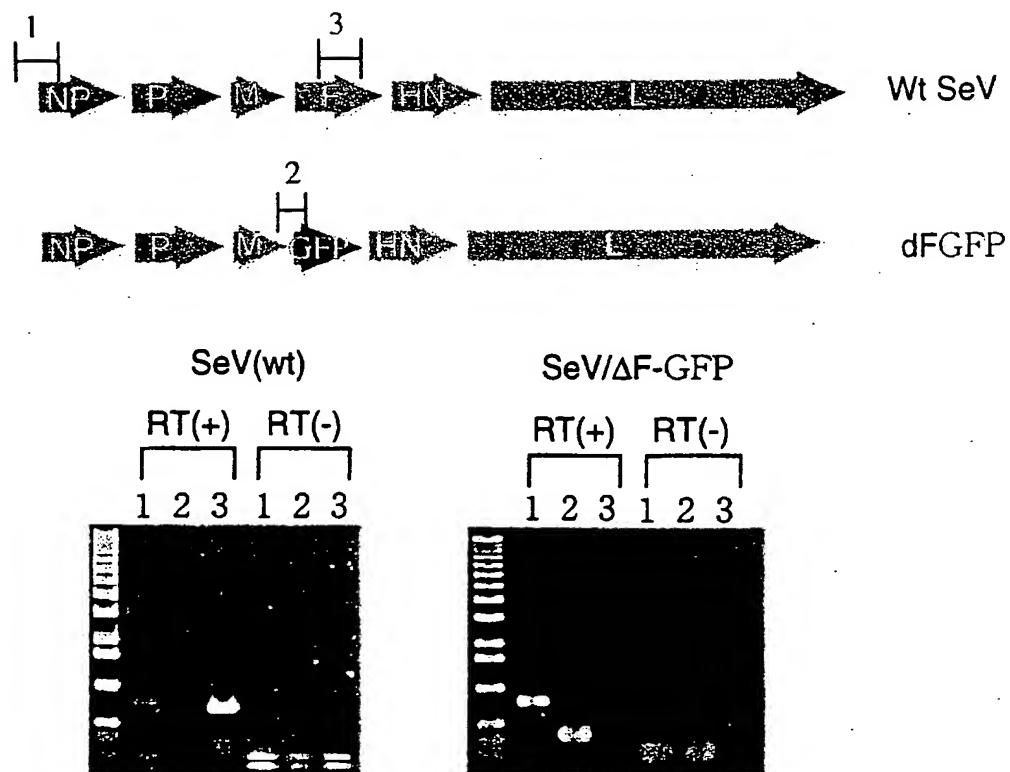
1 0 / 6 2

10



11 / 62

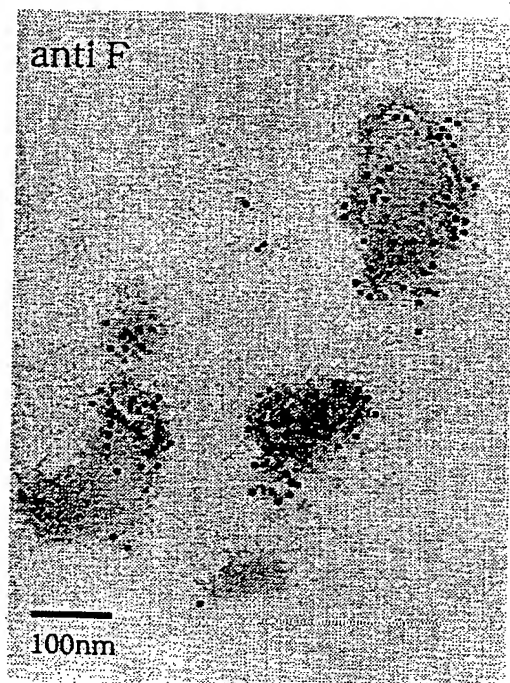
図 11



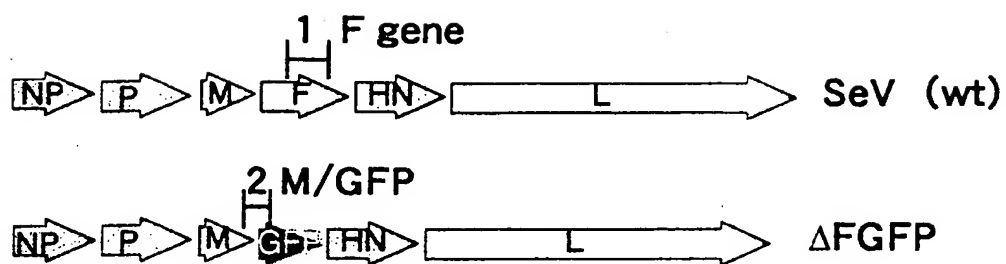
- 1: +18-NP、+18Not.Iサイトの存在の確認
- 2: M-GFP、GFP遺伝子がF遺伝子欠損部位の存在の確認
- 3: F gene、F遺伝子の存在の確認

12 / 62

図 12



1 3 / 6 2

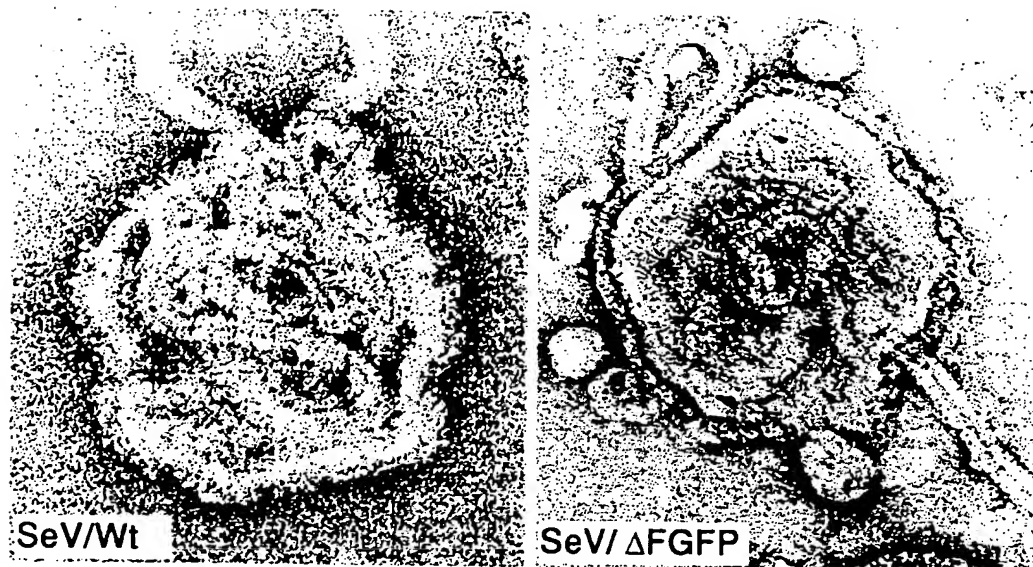
 1 3


Primer Set	ΔFGFP	F/ΔFGFP	GFP/SeV	SeV (wt)
1	-	+	+	+
2	+	+	-	-



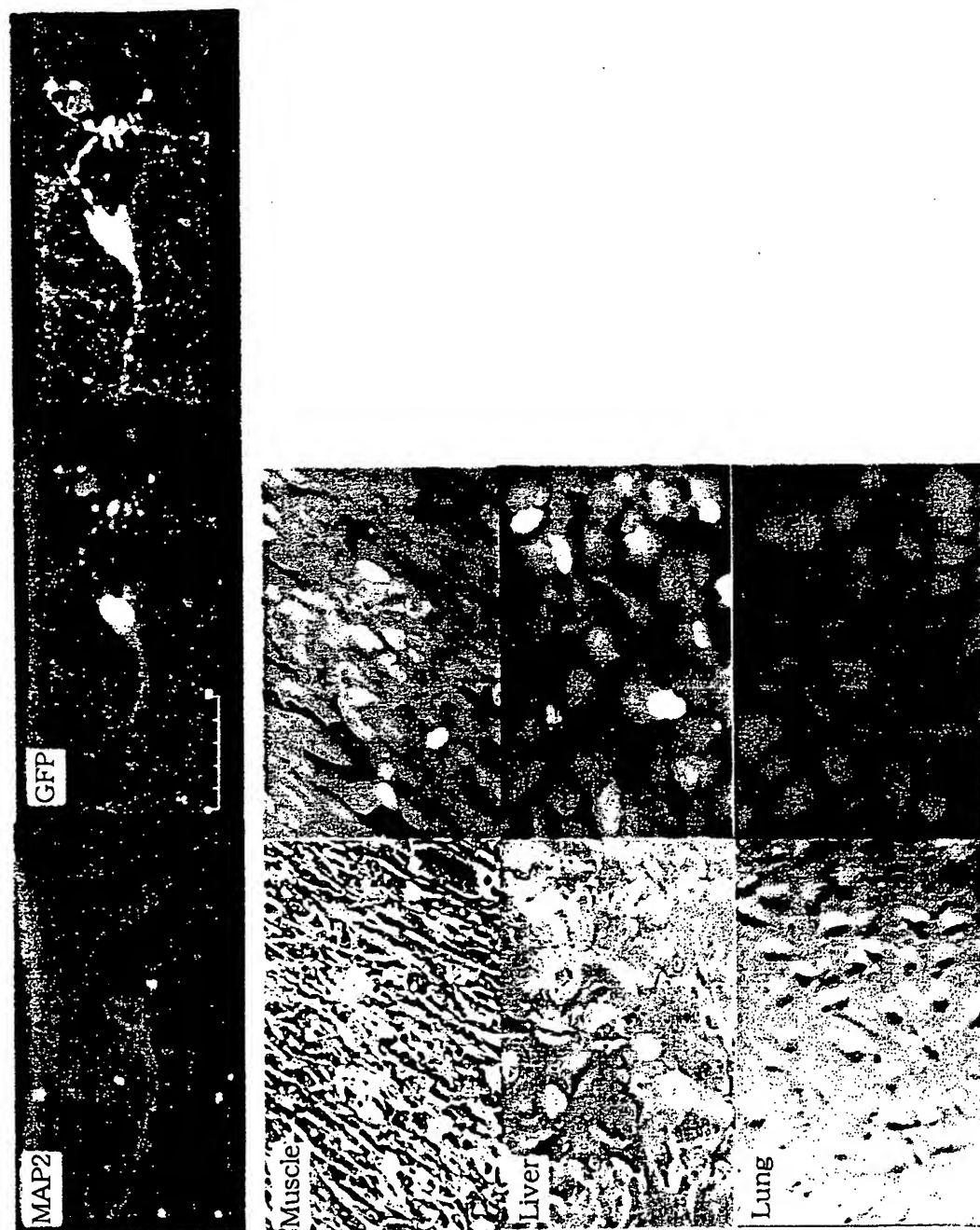
14/62

図 14



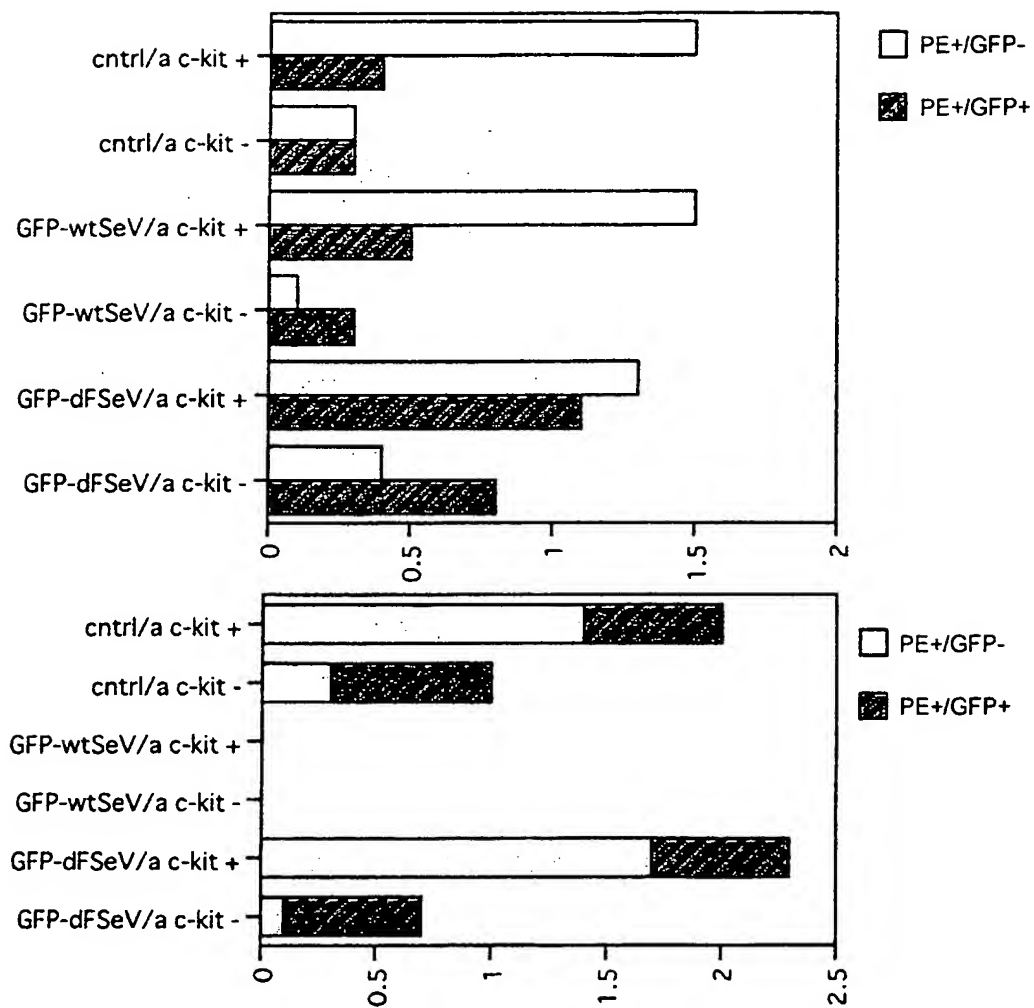
15 / 62

図 15



16 / 62

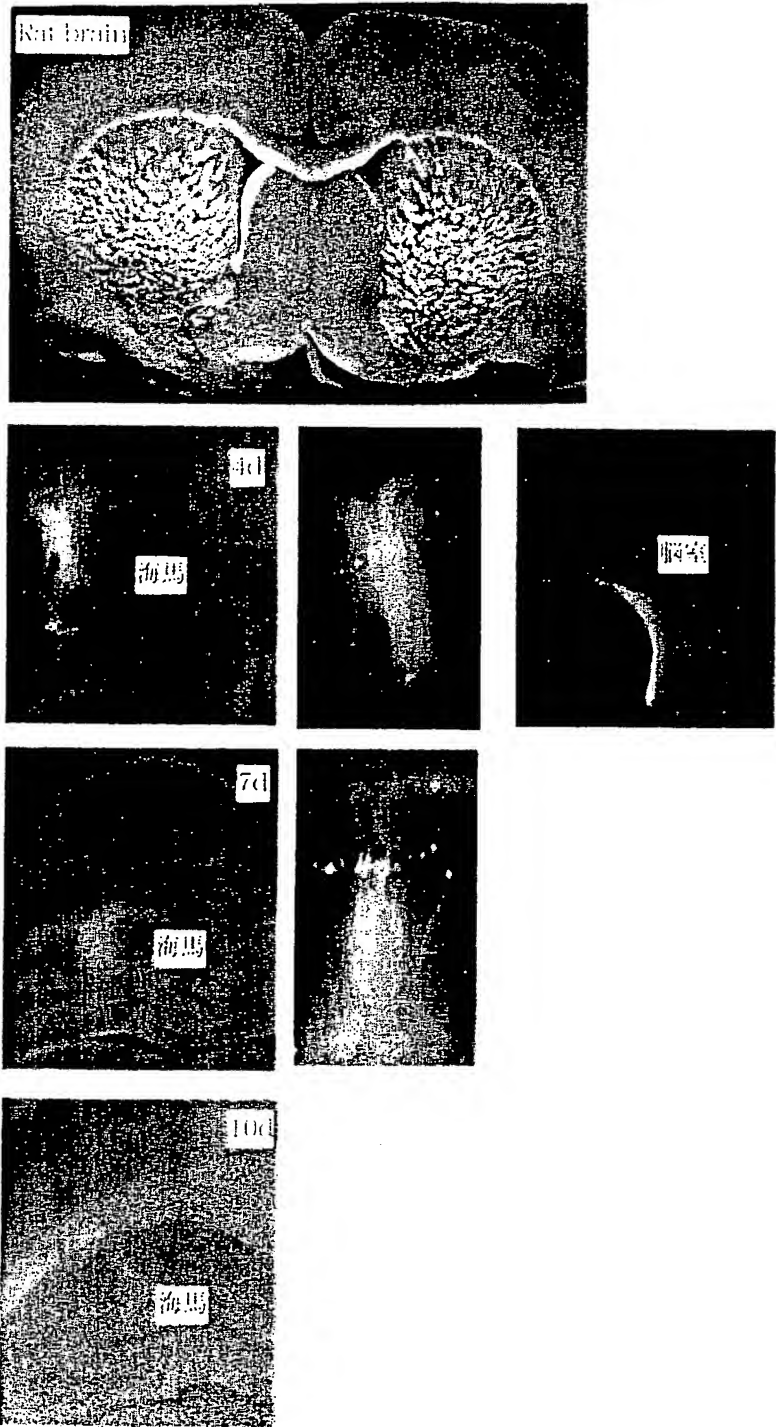
図 16



17 / 62

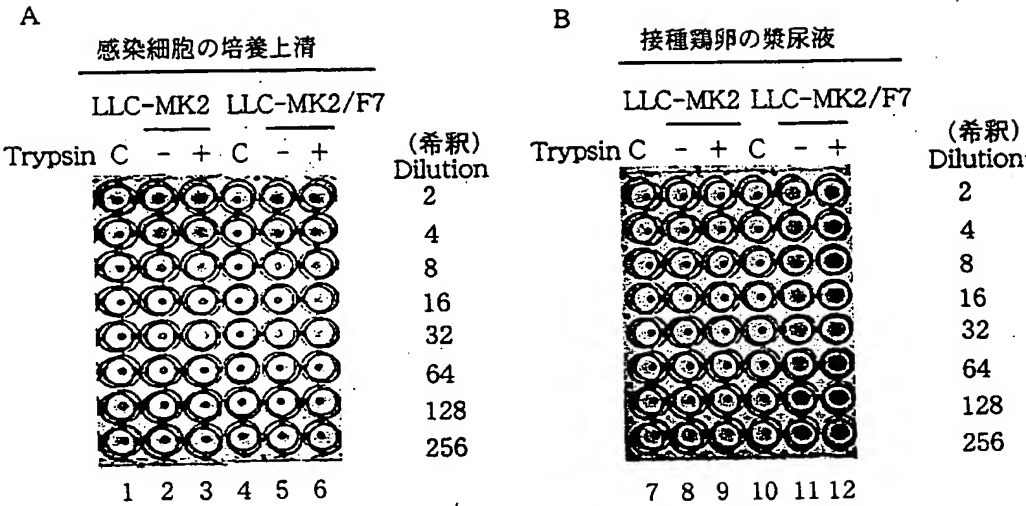
図 17

in vivo



18 / 62

図 18

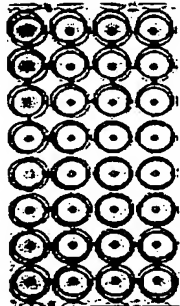


1 9 / 6 2

図 1 9

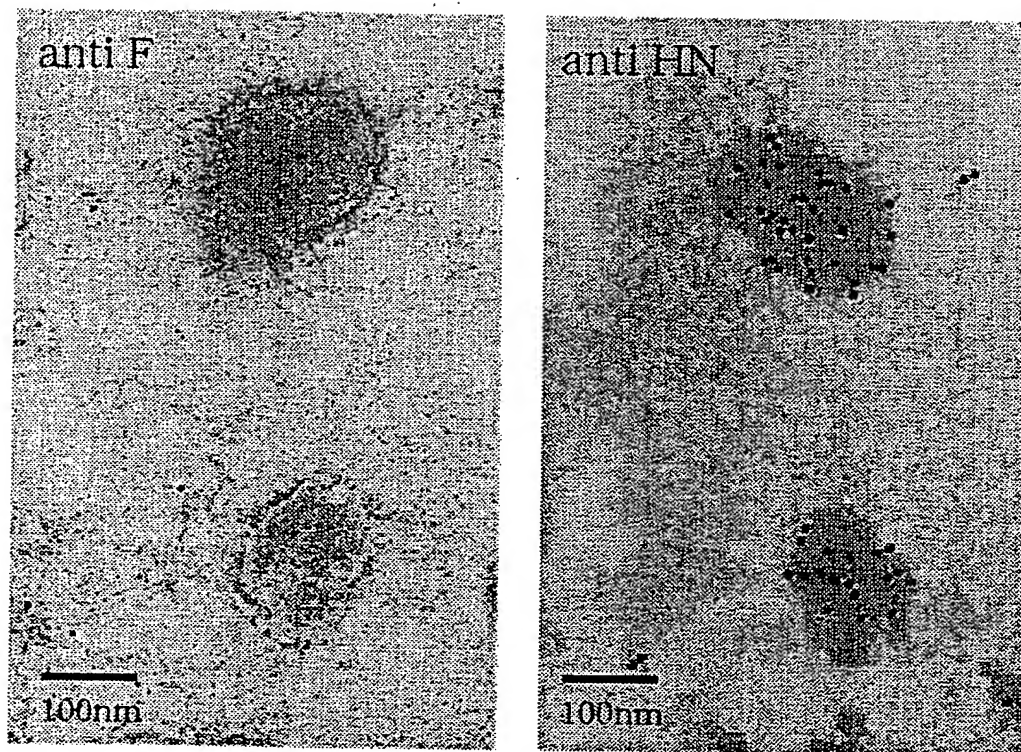
C

接種鶏卵の漿尿液 (P 2)

Dilution(希釈)			
	2	13: 陽性対象群、F発現細胞の培養上清(lane 6)接種した	
	4	14: 陰性対象群、PBS	
	8		
	16	15: HA陽性の漿尿液(lane 11)発育鶏卵に再接種した (P 2)	
	32		
	64	16: HA陽性の漿尿液(lane 12)発育鶏卵に再接種した (P 2)	
	128		
	256		
13 14 15 16			

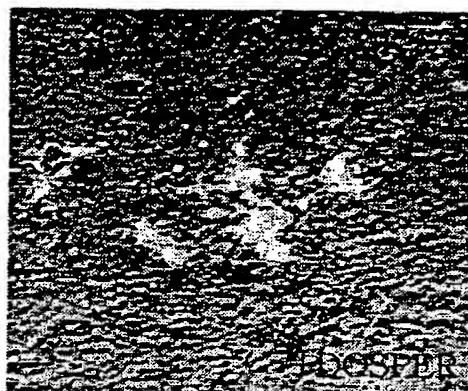
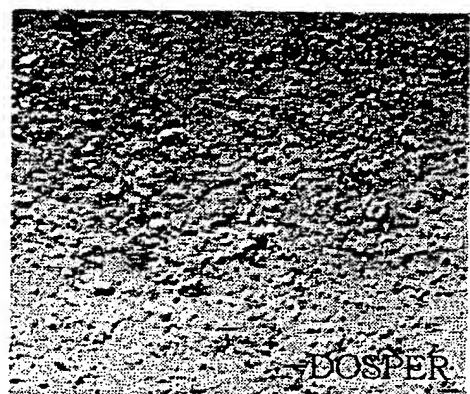
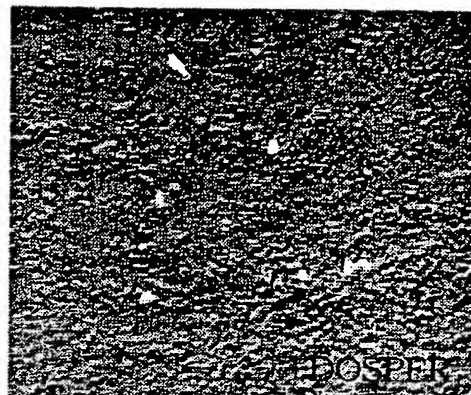
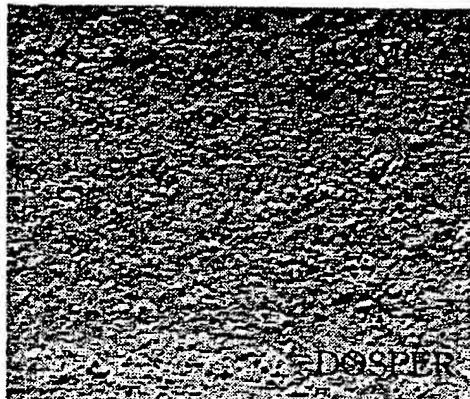
20 / 62

図 20



21/62

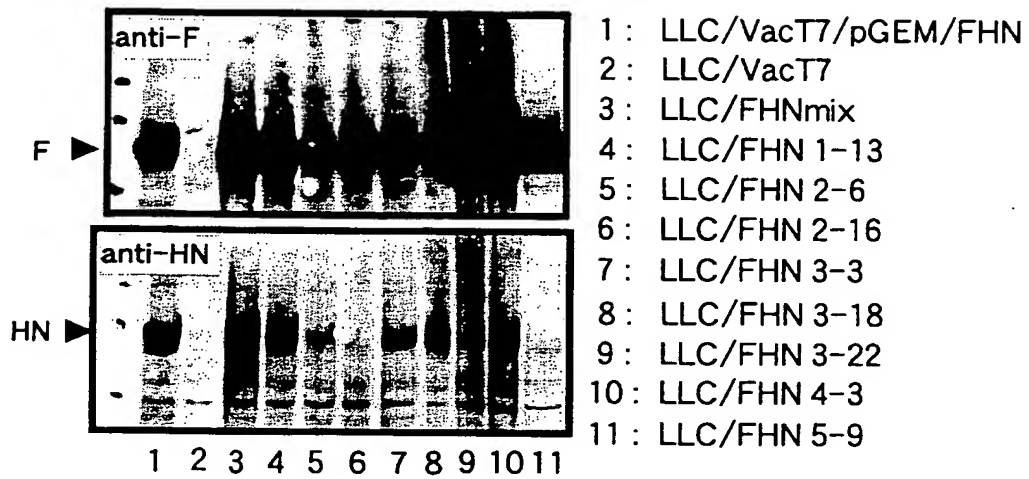
図 21





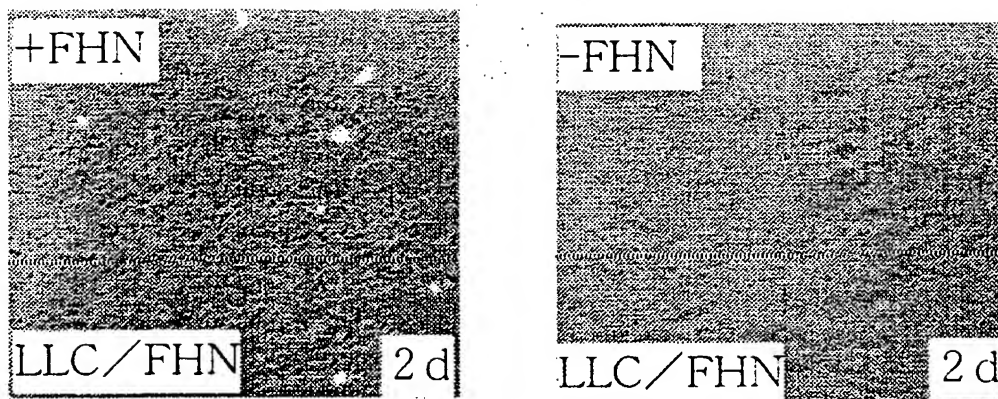
22 / 62

22

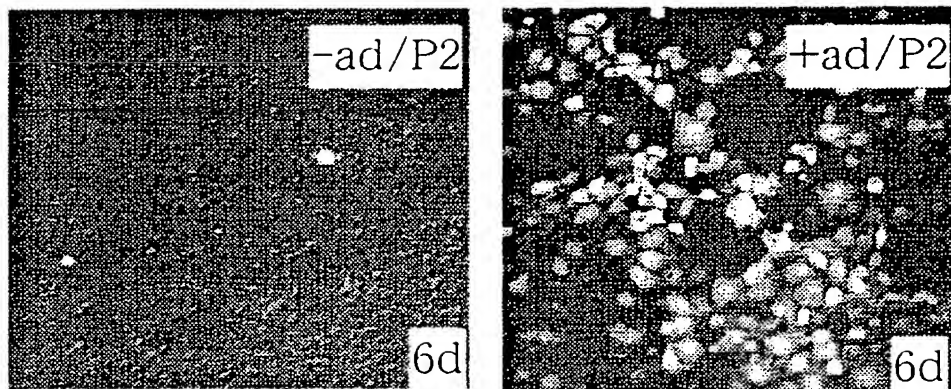


23 / 62

図 23

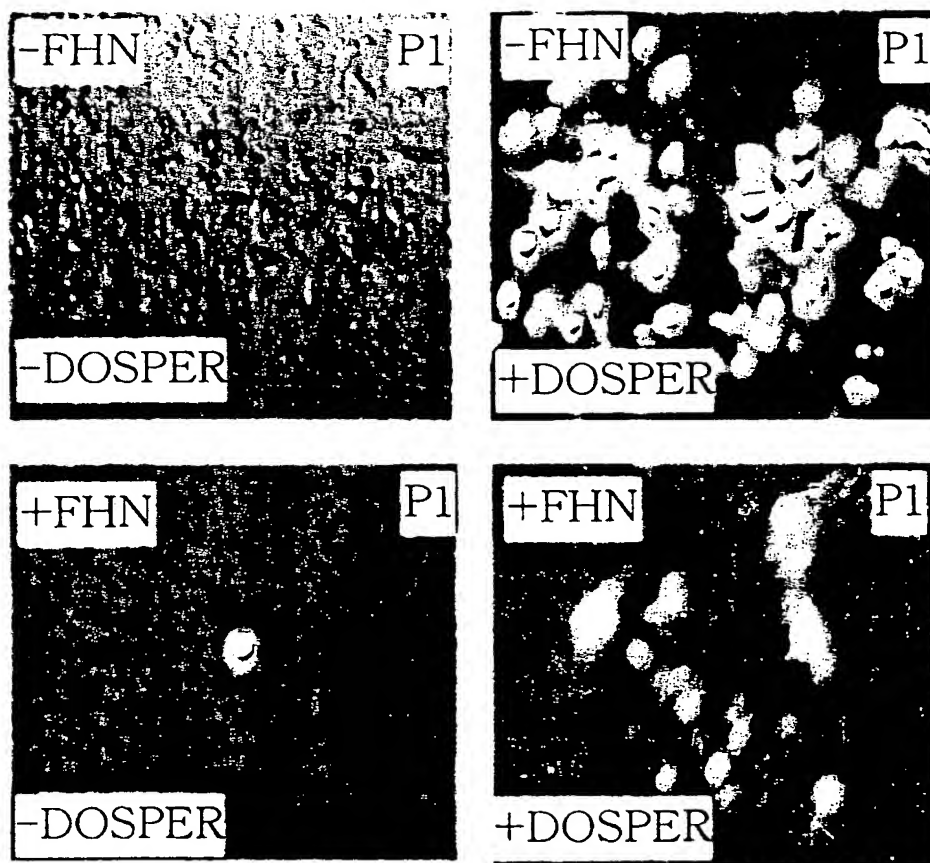


P1 sup重層

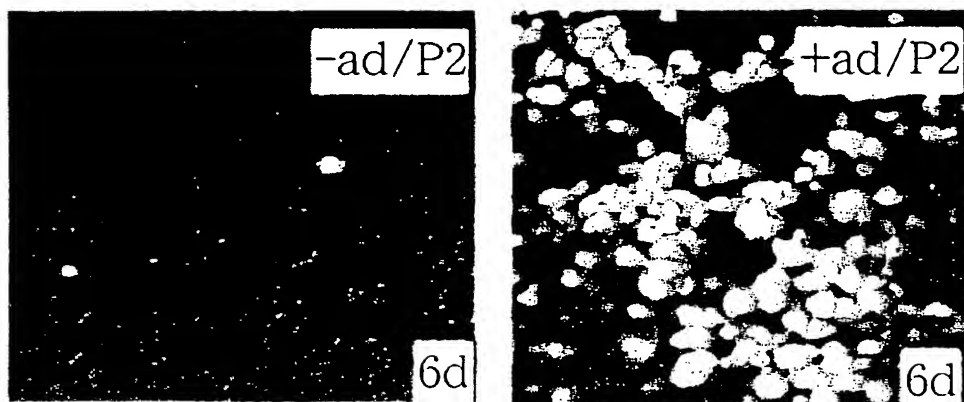


24/62

図 24

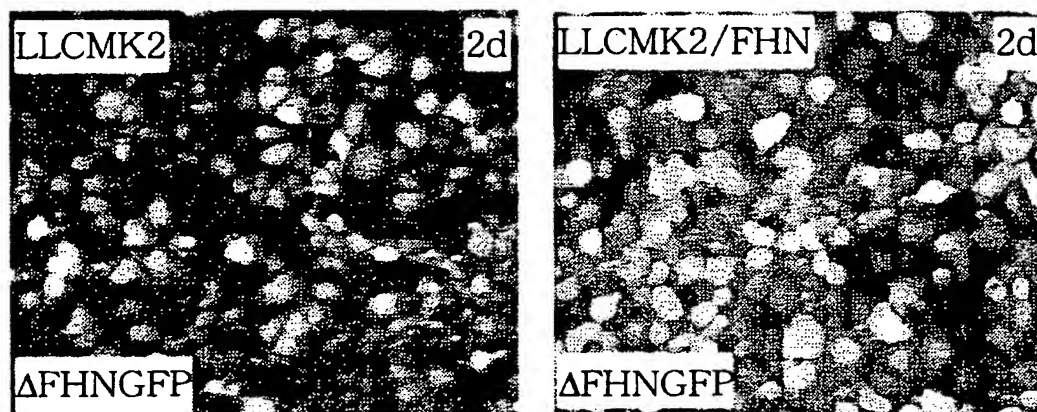


P1 sup重層



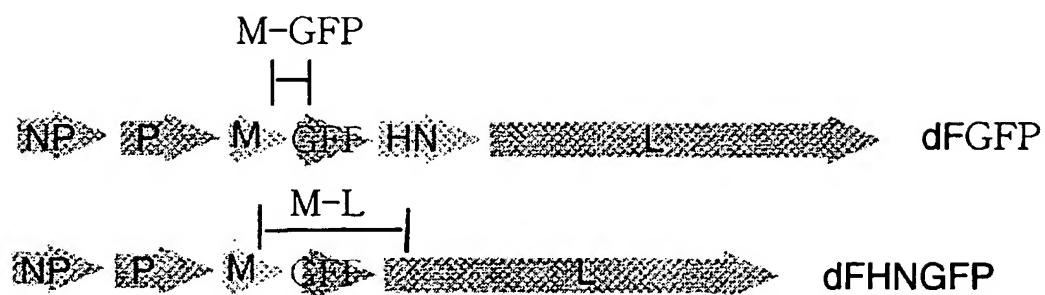
25 / 62

図 25

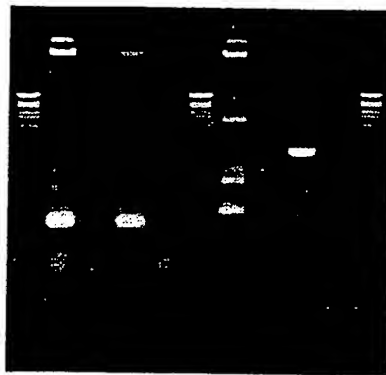


26 / 62

26



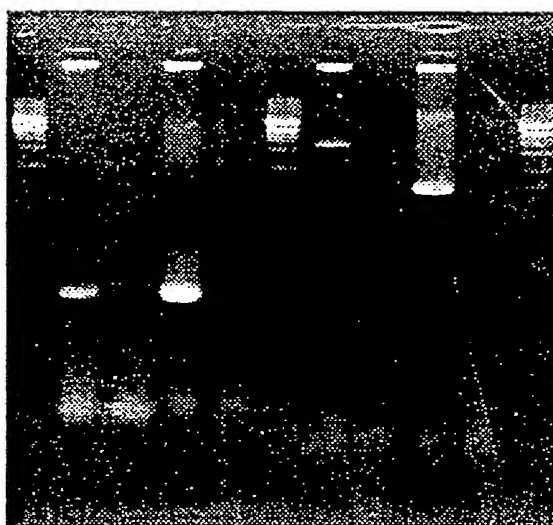
	M-GFP				M-L			
	$\Delta^F$		$\Delta^{FHN}$		$\Delta^F$		$\Delta^{FHN}$	
RT	+	-	+	-	+	-	+	-



27 / 62

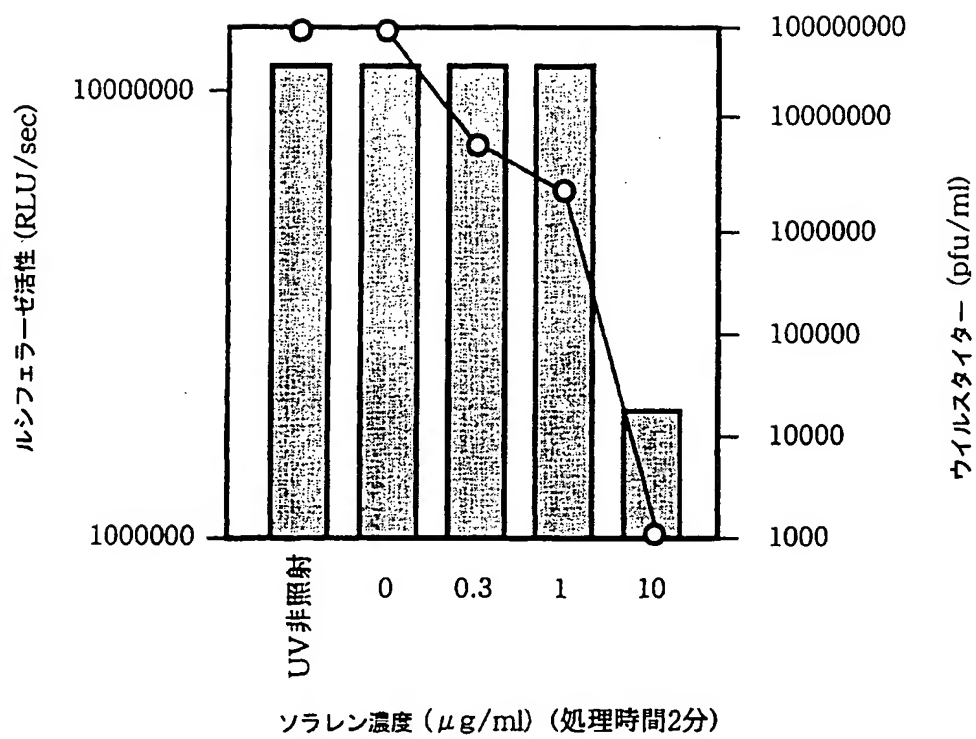
図 27

M-GFP		M-L	
$\Delta F$		$\Delta FHN$	
$\Delta F$		$\Delta FHN$	
RT	+-	+-	+-



28/62

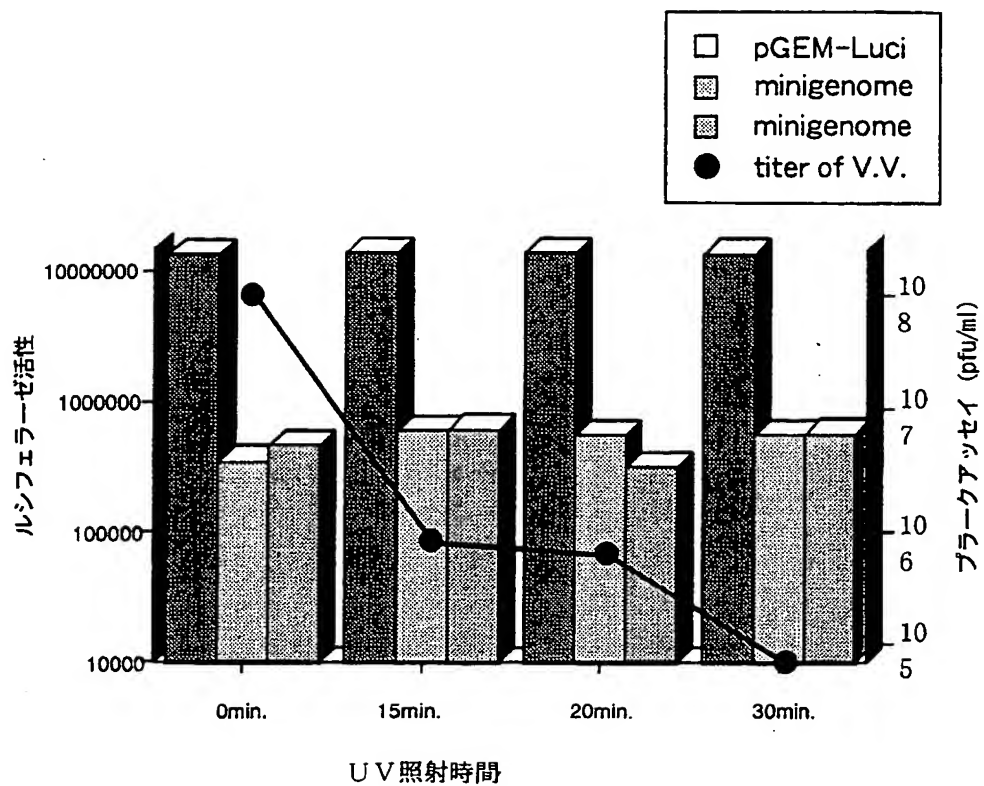
図 28



○印 ウイルスタイター

29 / 62

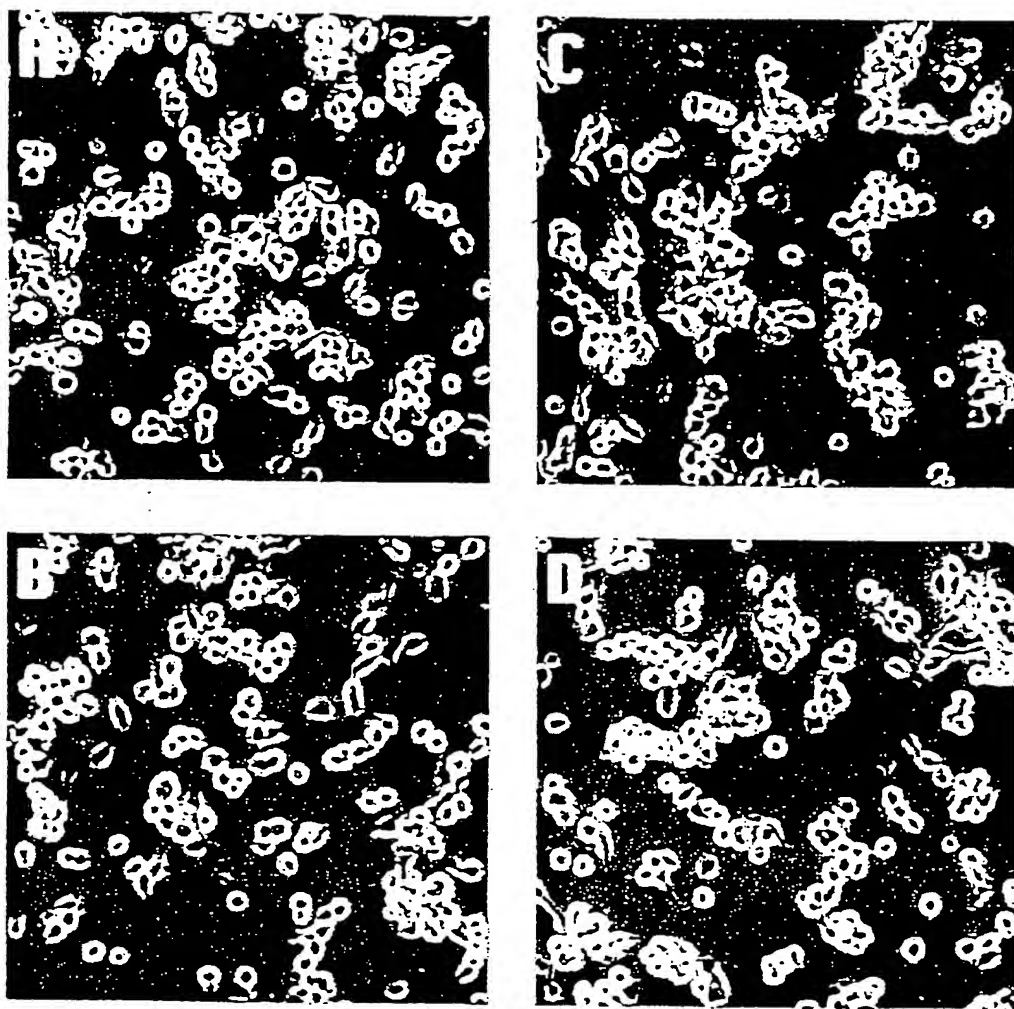
図 29





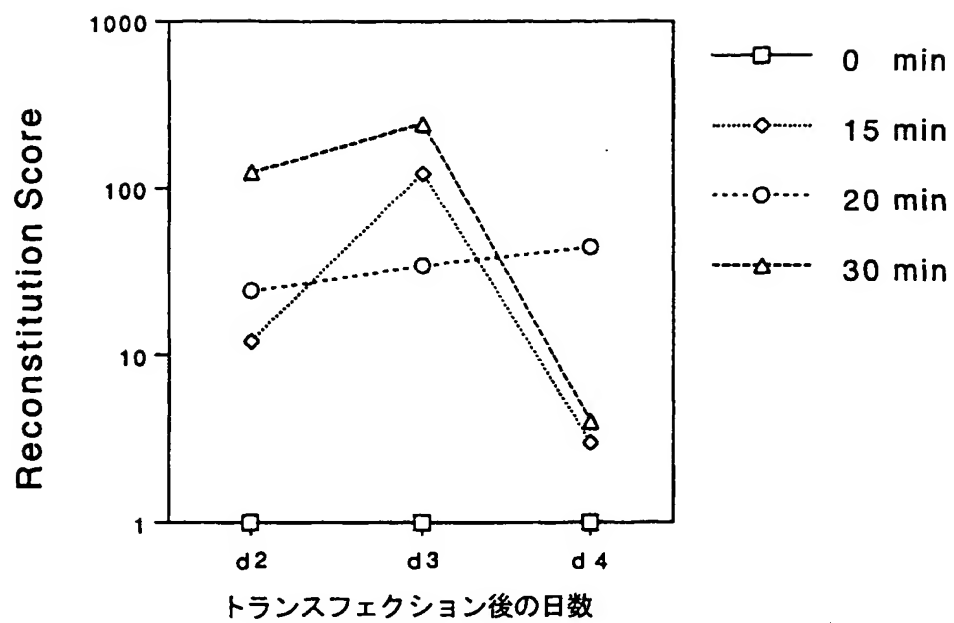
30 / 62

図 30



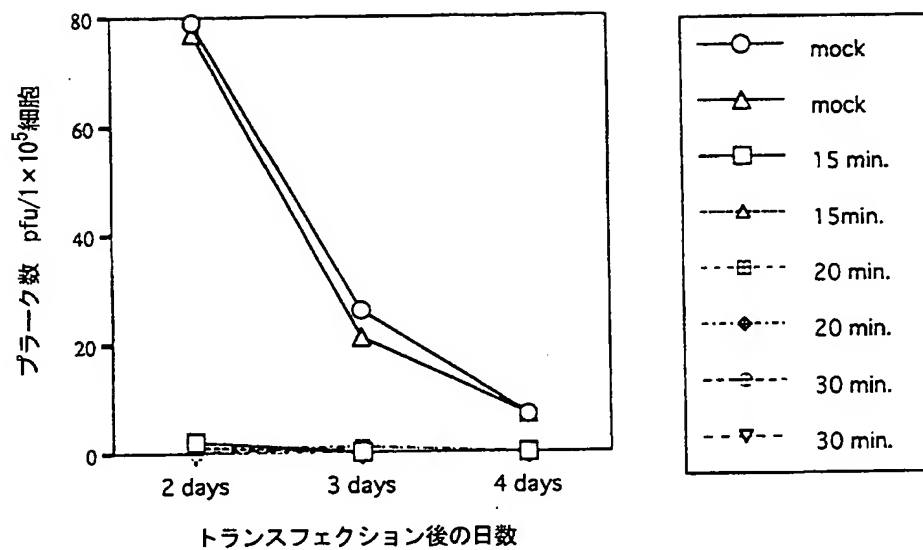
31/62

図 31



32 / 62

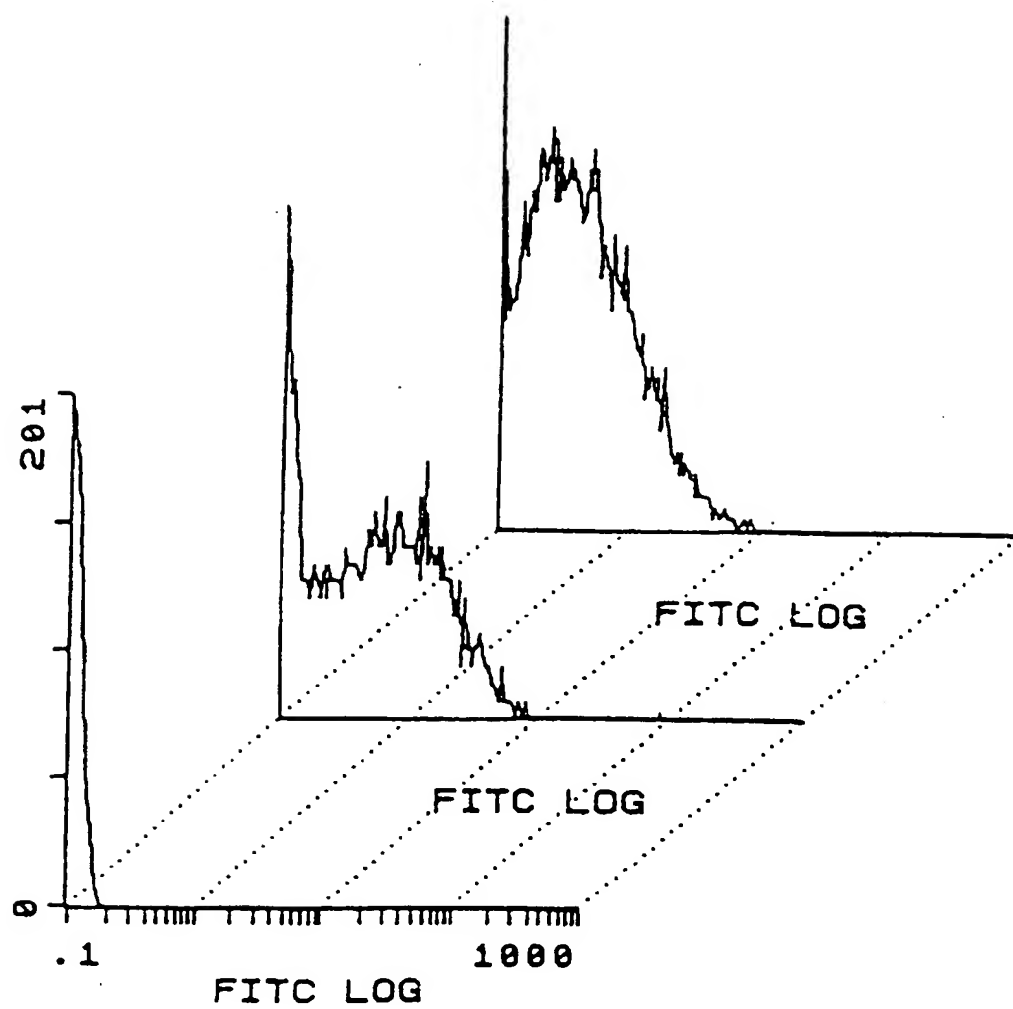
図 3 2





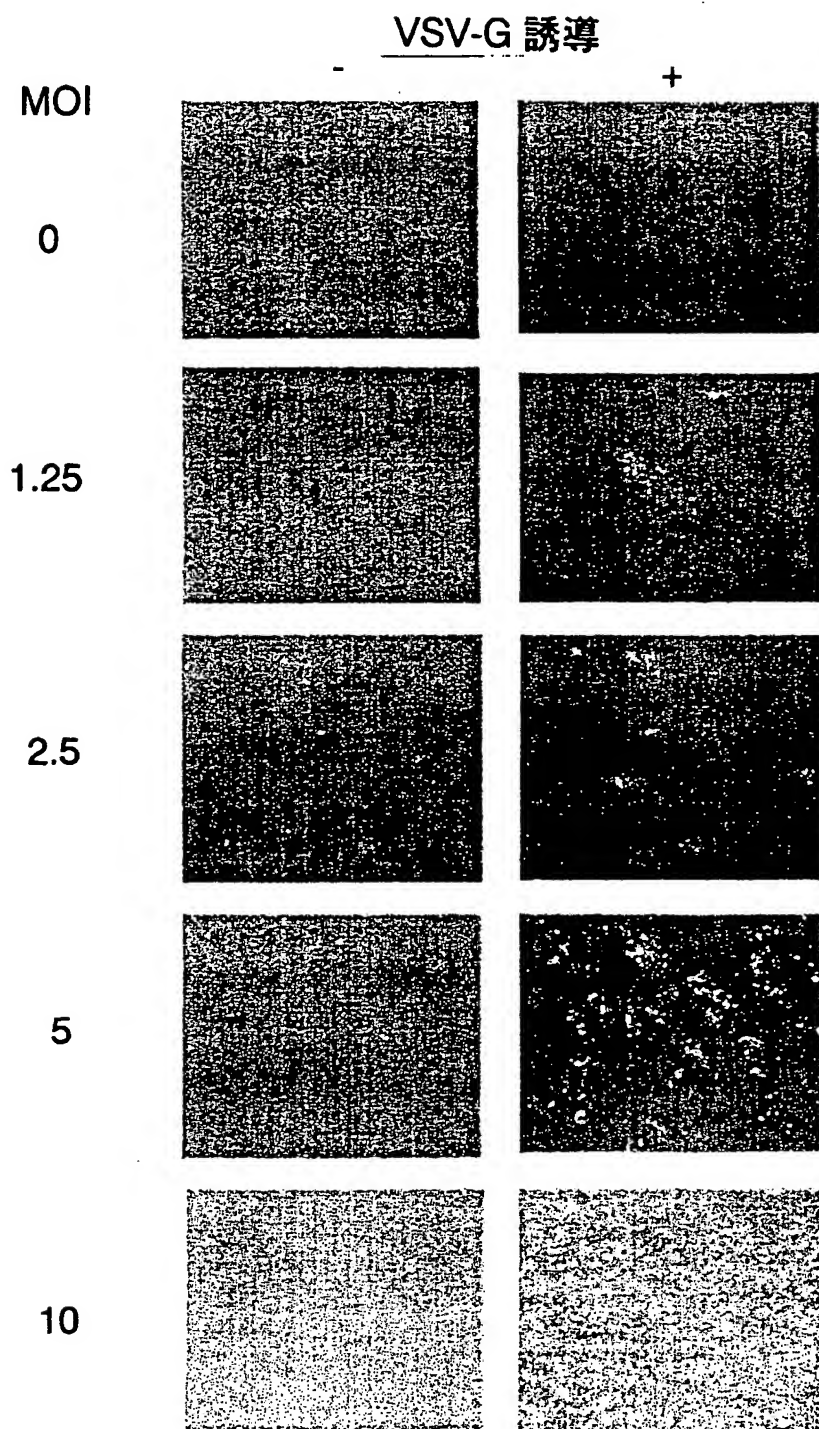
34/62

図34



35 / 62

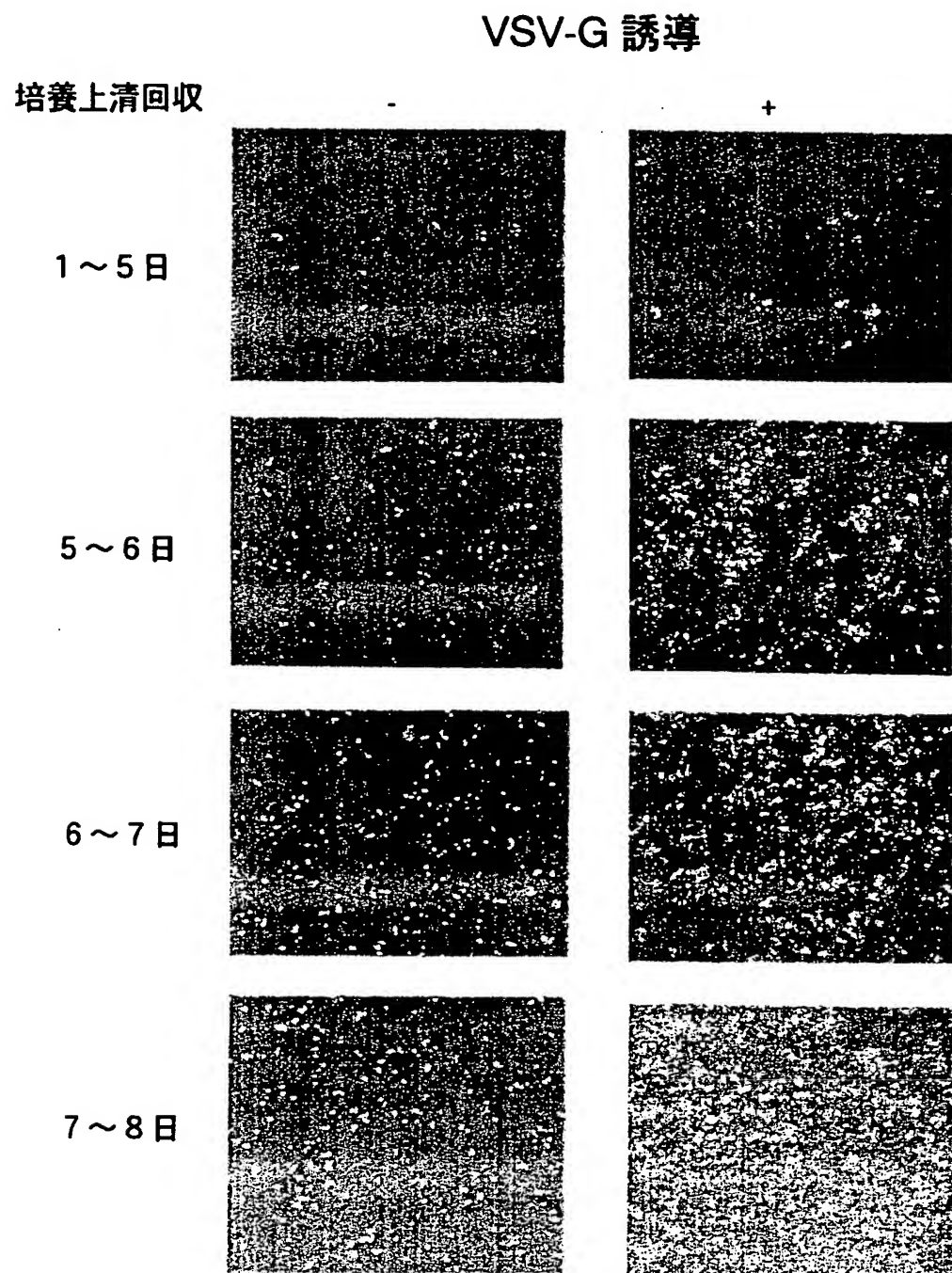
図 35



P3 ウイルス液 : 感染 7 ~ 8 日目培養上清

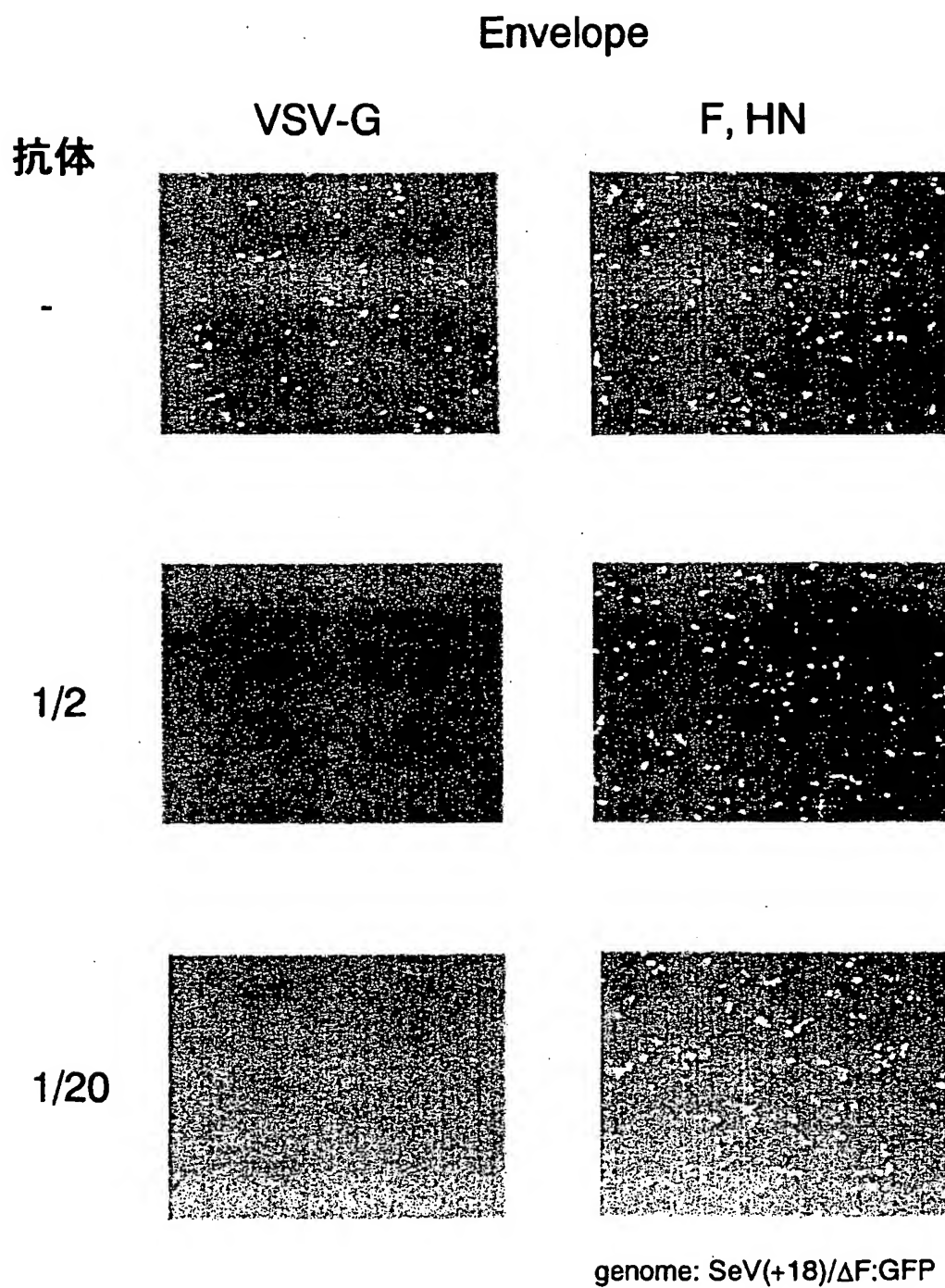
36 / 62

図 36



37 / 62

図 37

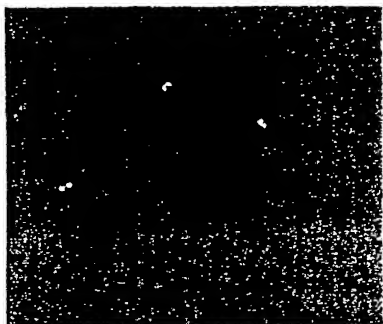




38 / 62

☒ 38

- VSV-G



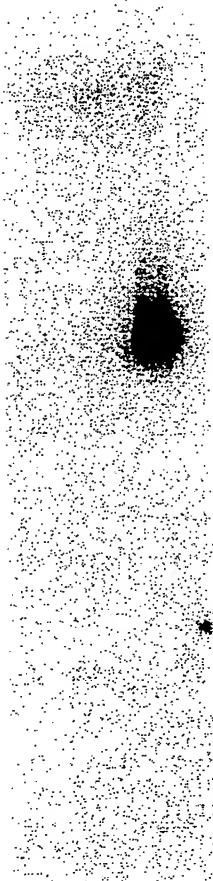
+ VSV-G



39 / 62

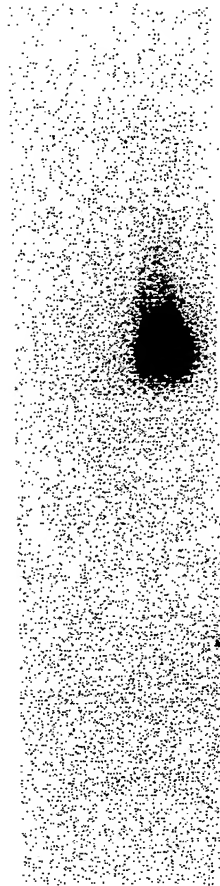
☒ 39

1 2 3



anti-F

1 2 3



anti-HN

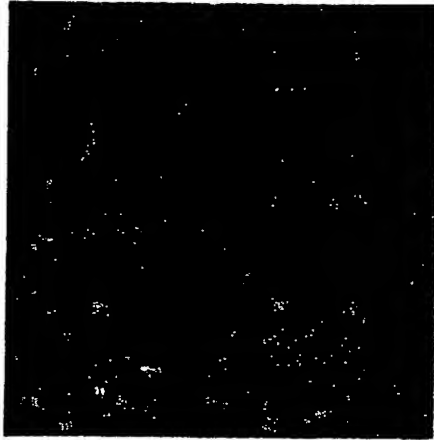
1 2 3



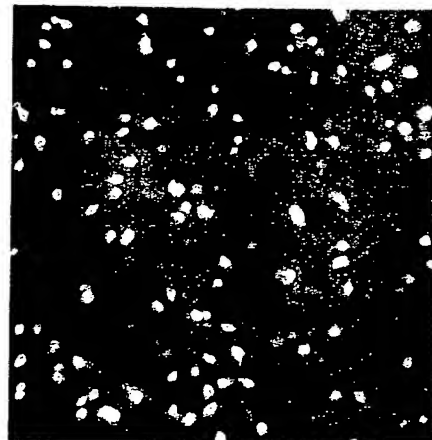
anti-SeV

40 / 62

図 40



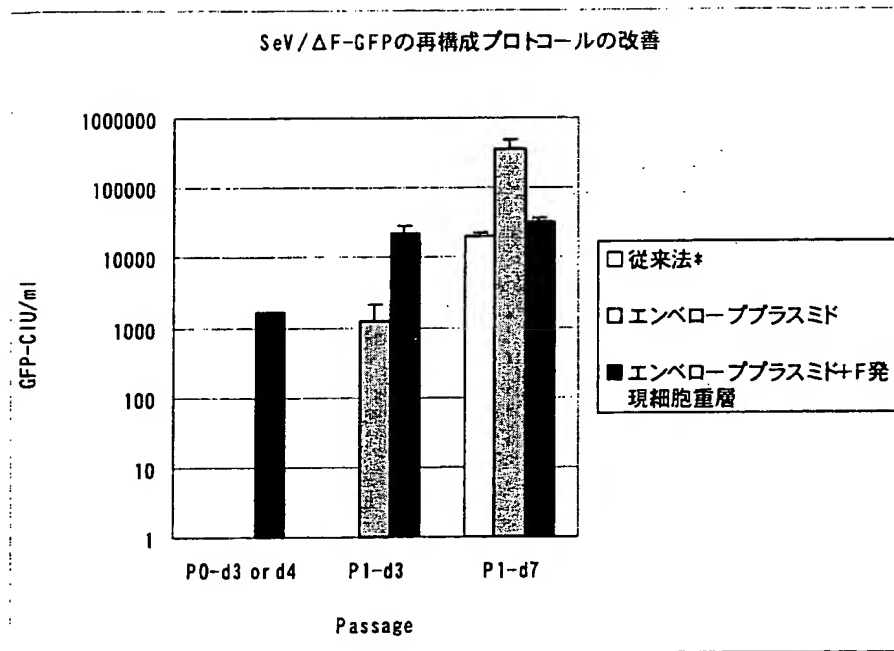
抗 SeV 抗体および抗ウサギ蛍光標識  
二次抗体を用いた CIU 測定



GFP 発現細胞による CIU 測定  
(対照)

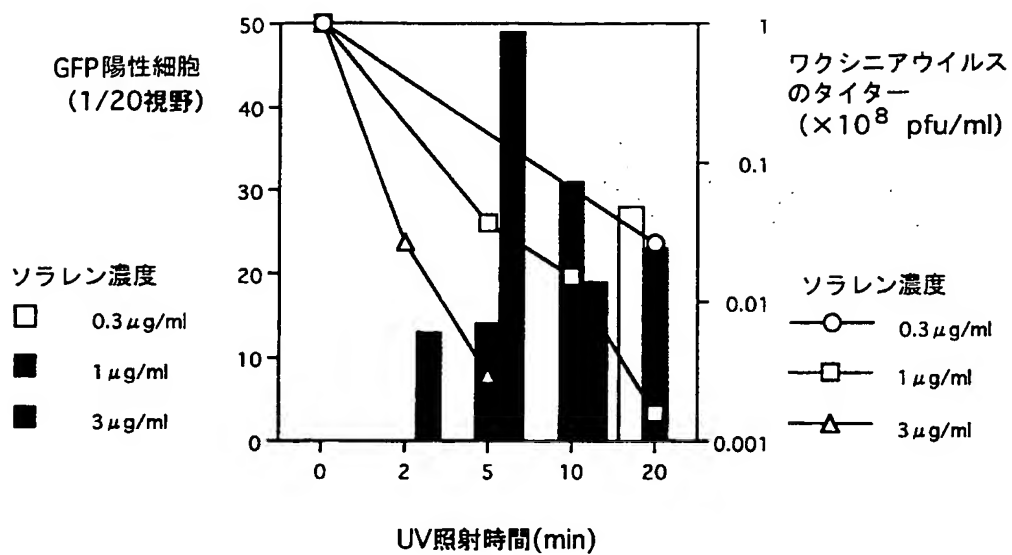
41/62

図 4 1



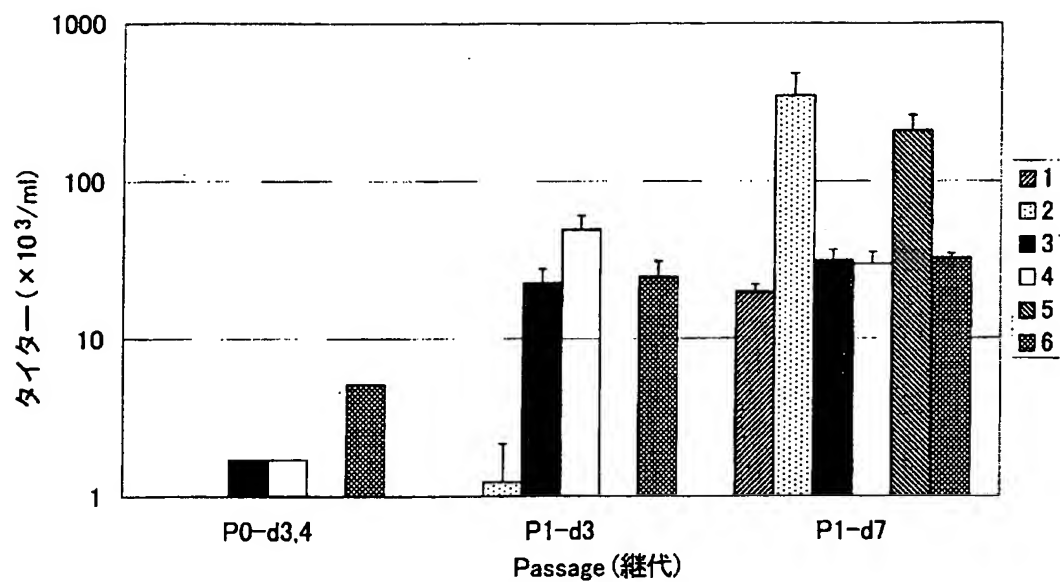
42 / 62

図42



43 / 62

図 4 3



	V.V	V.V Lot.No	pGEM-FHN	O.L	P1-Tf
1	vTF7-3(3,5) $\phi$ 35mm*	990913	-	-	Dosper
2	vTF7-3(3,5) $\phi$ 35mm*	990913	○	-	O.L
3	vTF7-3(3,5) $\phi$ 35mm*	990913	○	○	O.L
4	vTF7-3(3,5) $\phi$ 35mm*	000202	○	○	O.L
5	vTF7-3(0,0)	990913	○	○	O.L
6	vTF7-3(3,5) $\phi$ 100mm*	000202	○	○	O.L

\*: PLWUV処理に使用したdishサイズ  
 () 内: (ソラレン濃度  $\mu\text{g}/\text{ml}$ , UV時間min)

44/62

44

Exp. No.

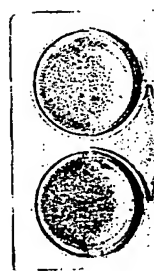
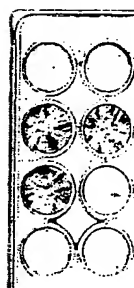
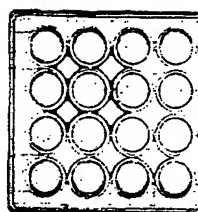
P1

P2

P3

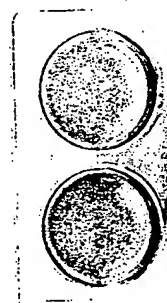
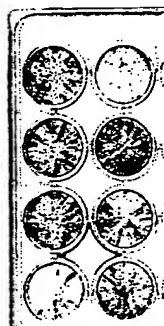
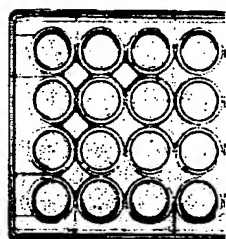
P4

1



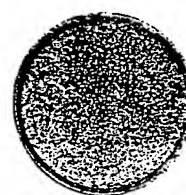
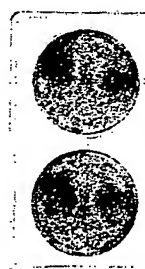
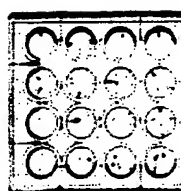
N. T.

2



N. T.

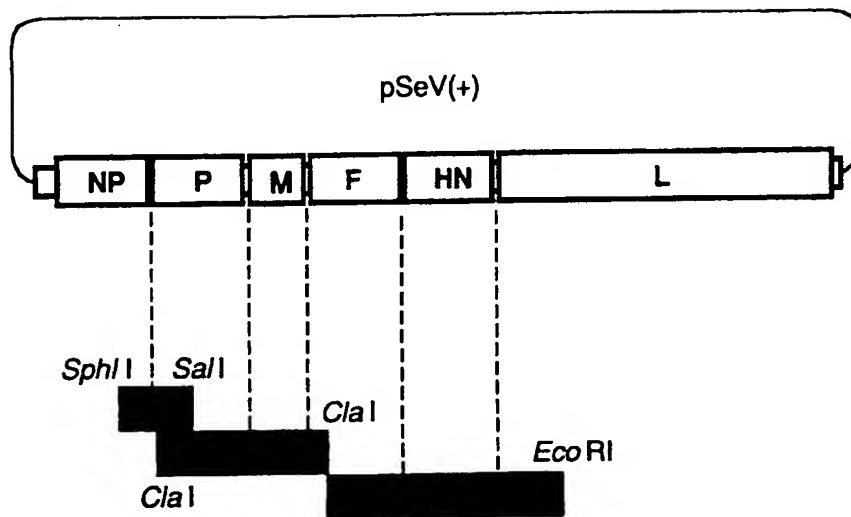
3



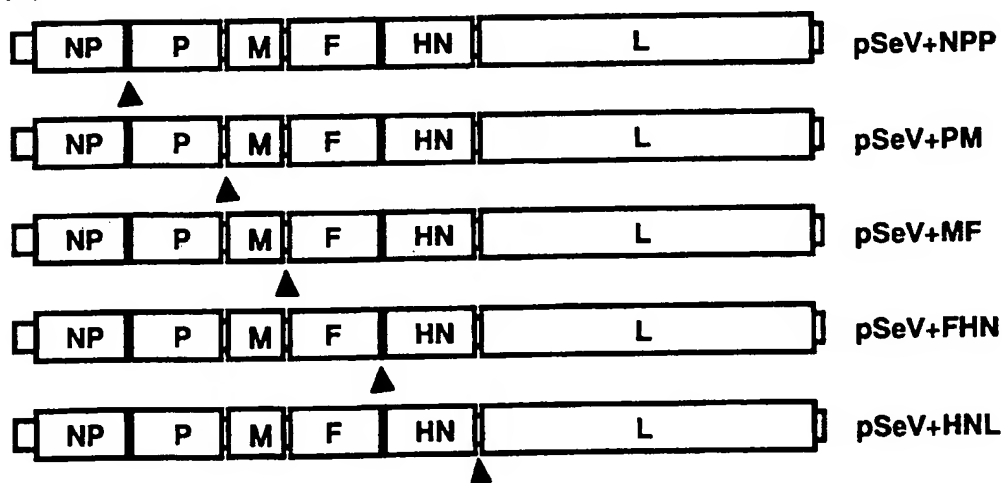
45 / 62

図 45

(A)



(B)

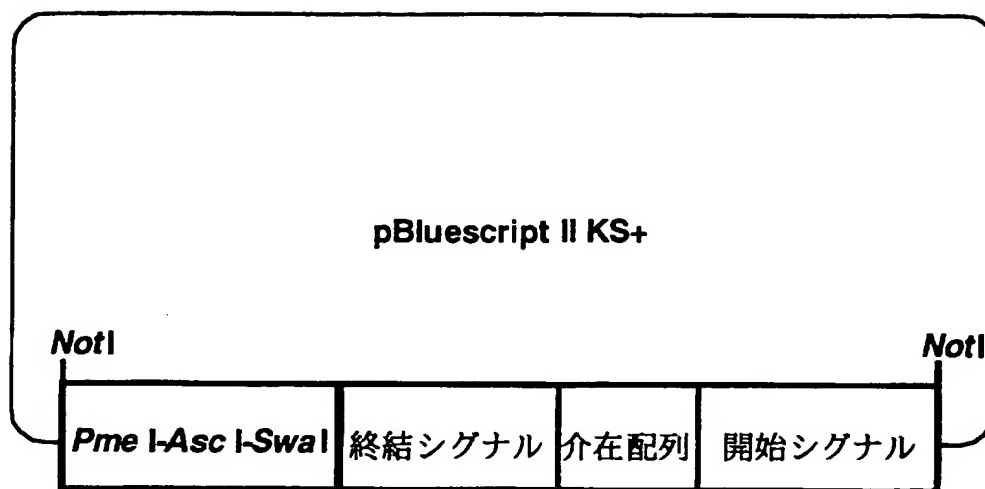


▲ 新しい Not I 部位



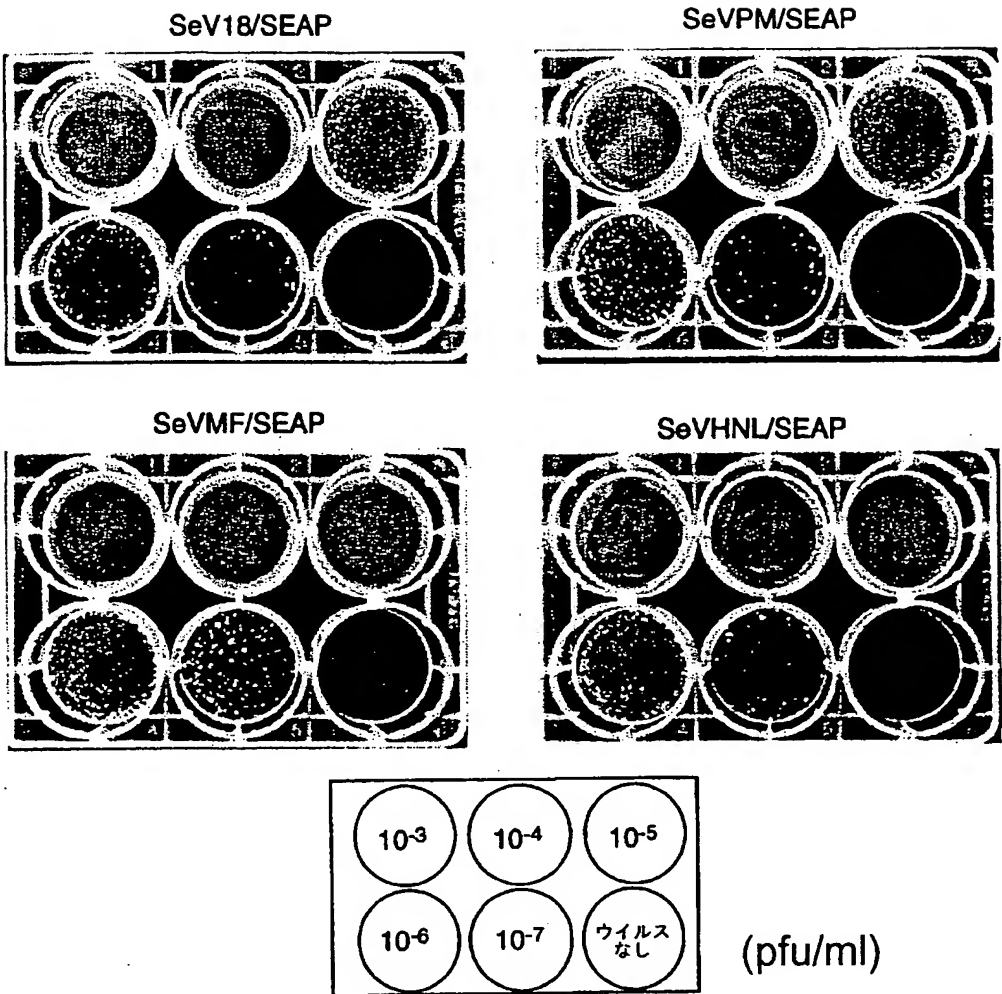
46 / 62

図 46



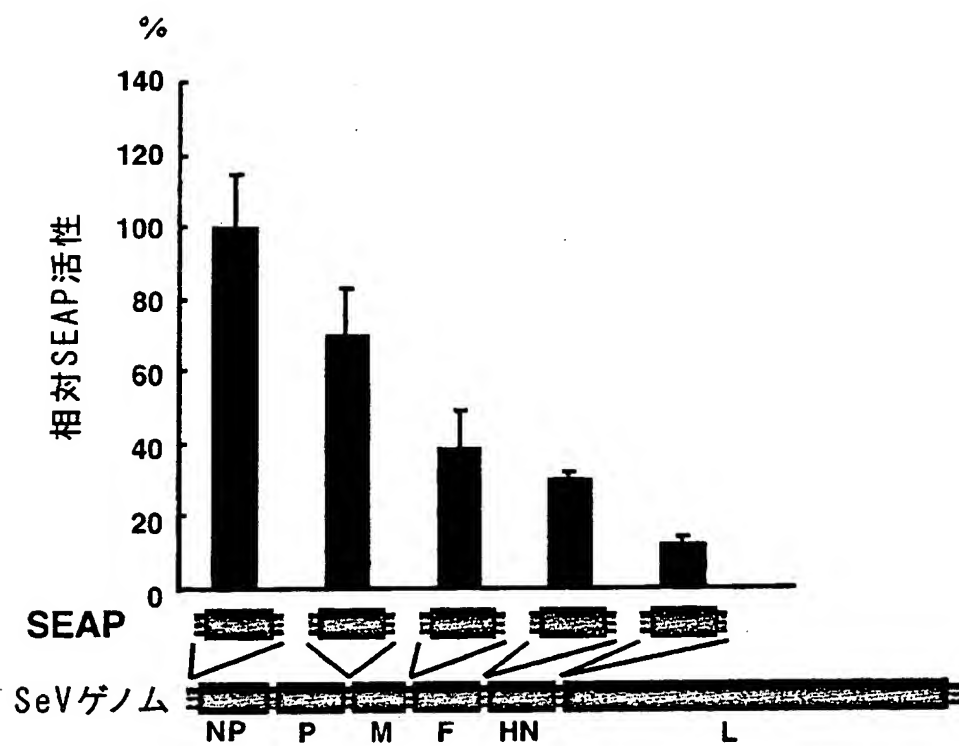
47 / 62

図 47



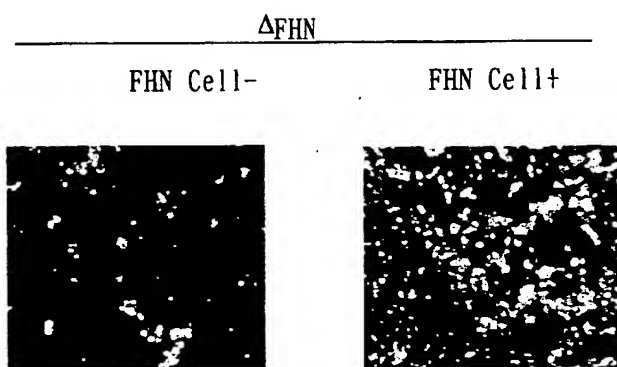
48 / 62

図 48



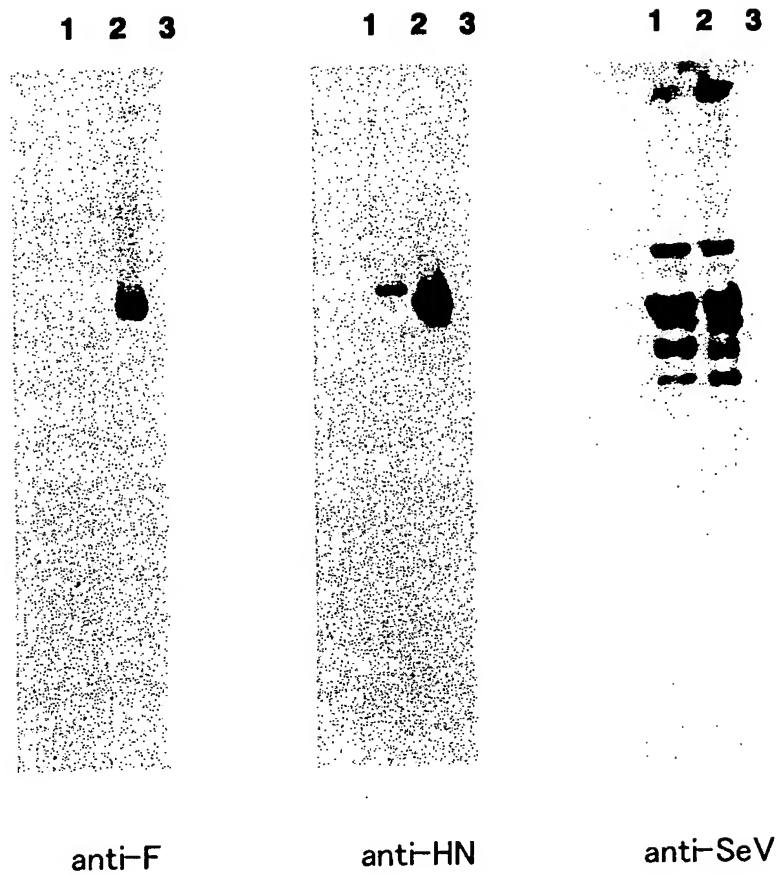
49 / 62

☒ 49



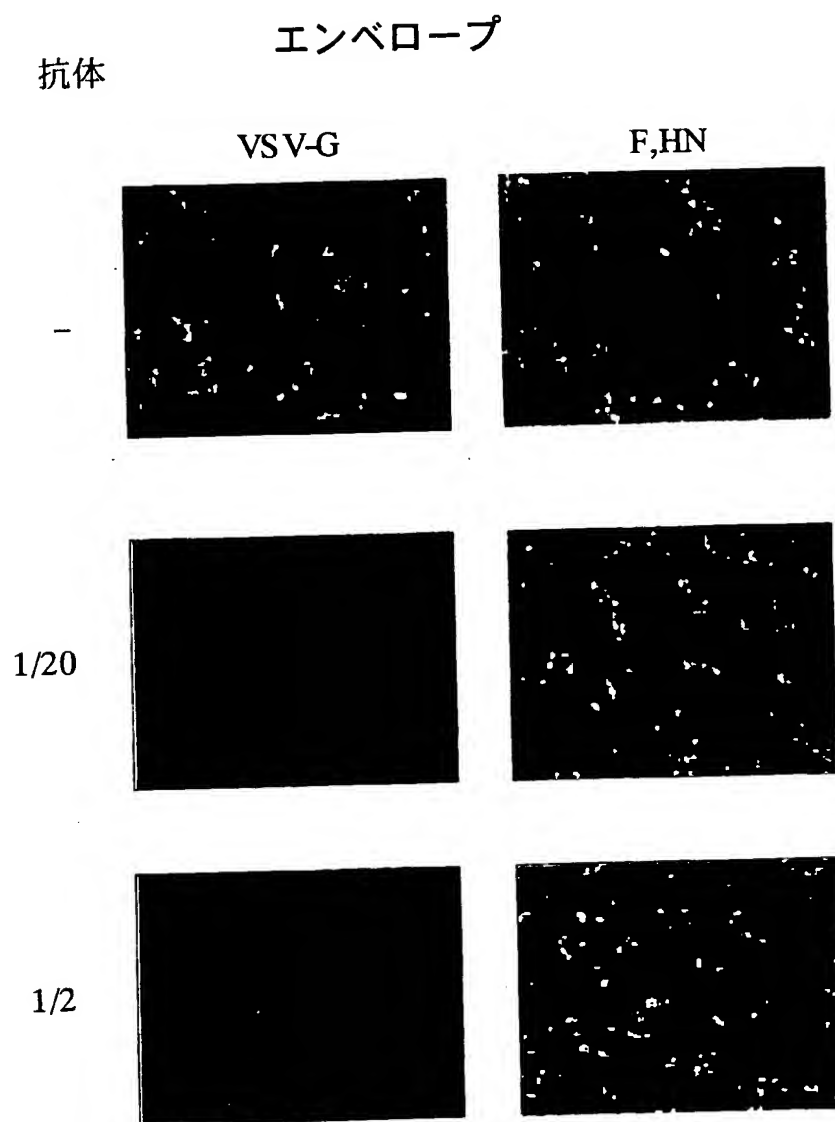
50 / 62

図 50



51 / 62

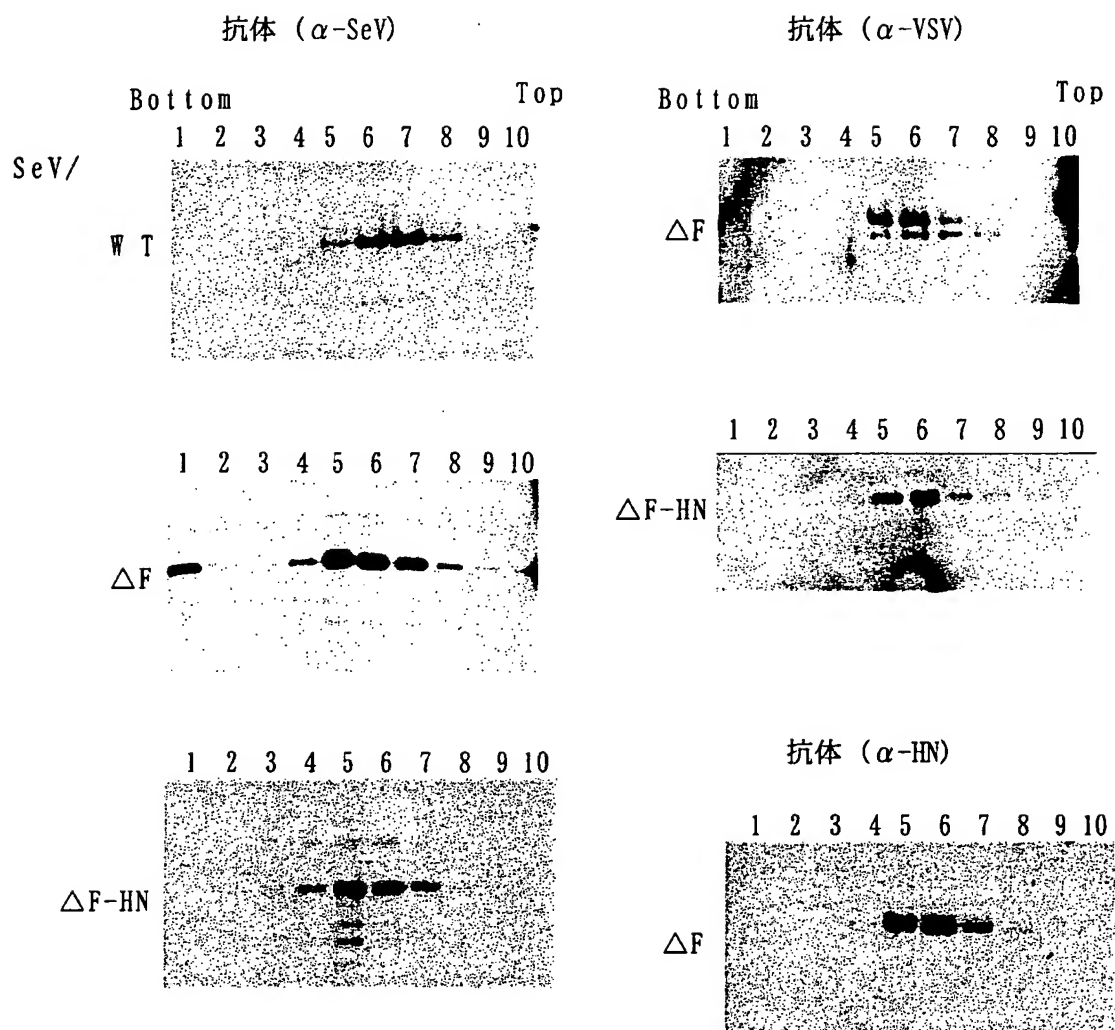
図 5 1



ゲノム: SeV(+18)/ΔF-HN:GFP

52/62

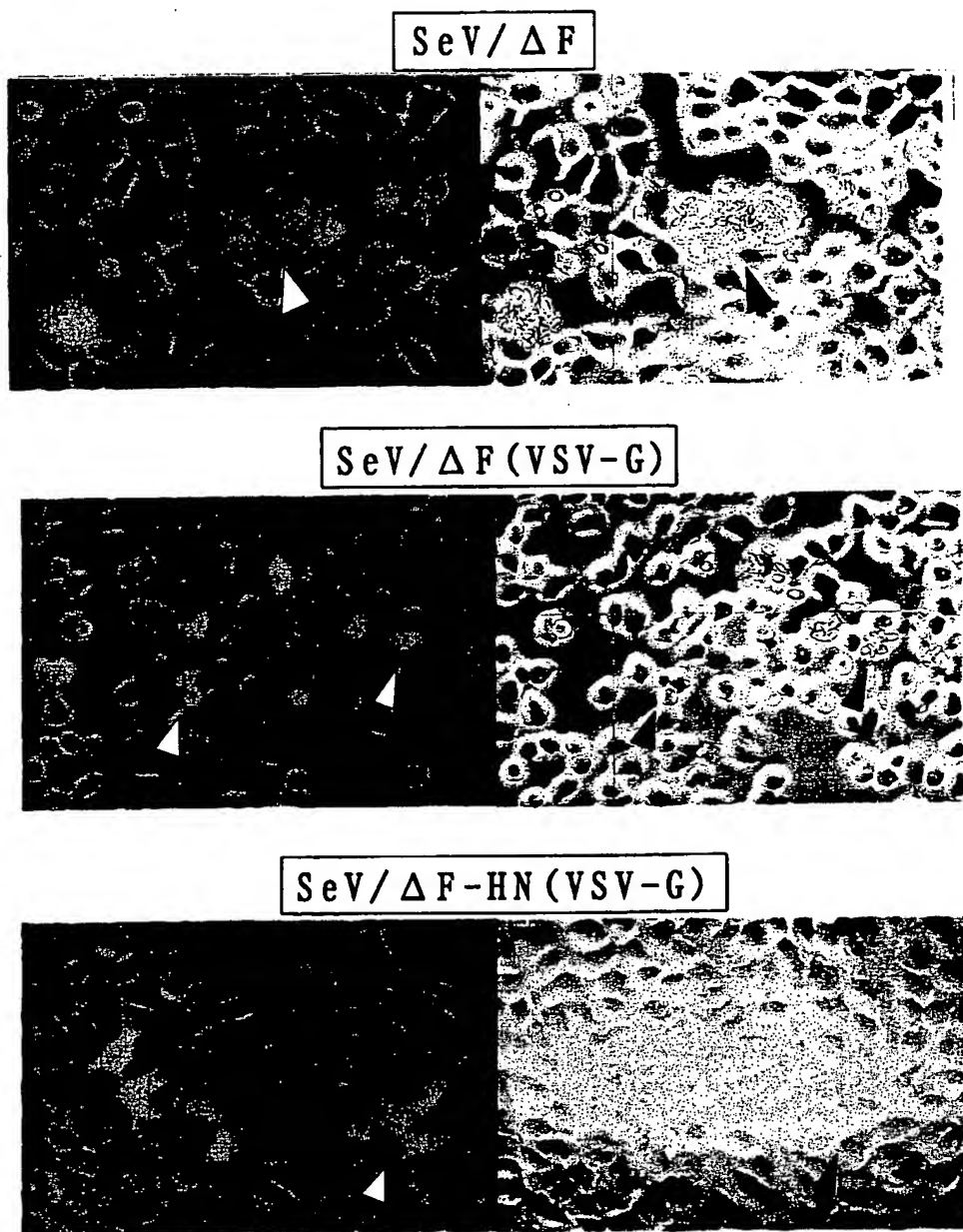
図 5 2



シヨ糖 : 20-60% リニアグラディエント

53 / 62

図 53

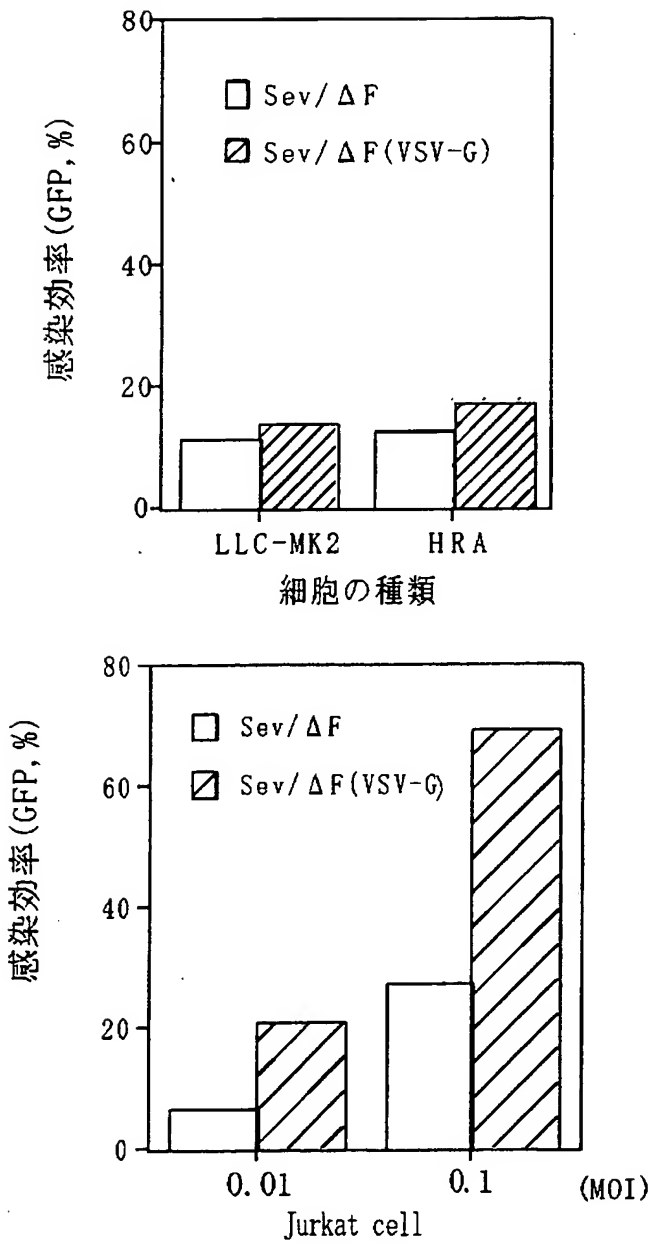


(LLC-MK2細胞)



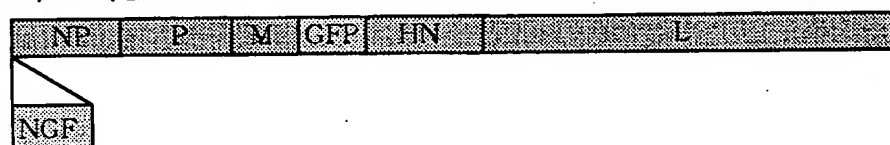
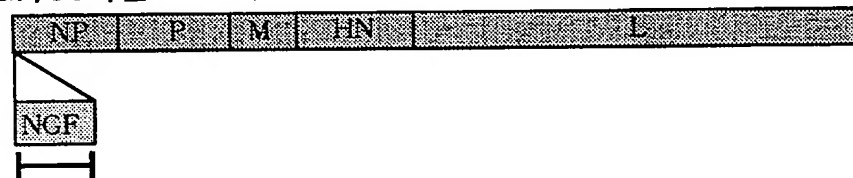
54 / 62

図 5 4

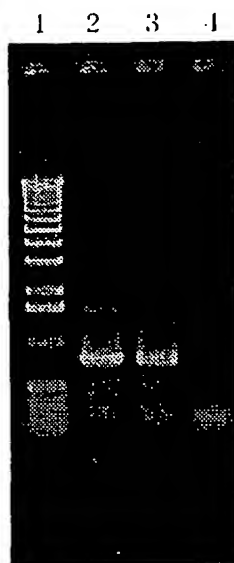


55 / 62

図 55

**pSeV18<sup>+</sup>b(+)****NGF/SeV/ΔF-GFP****NGF/SeV/ΔF**

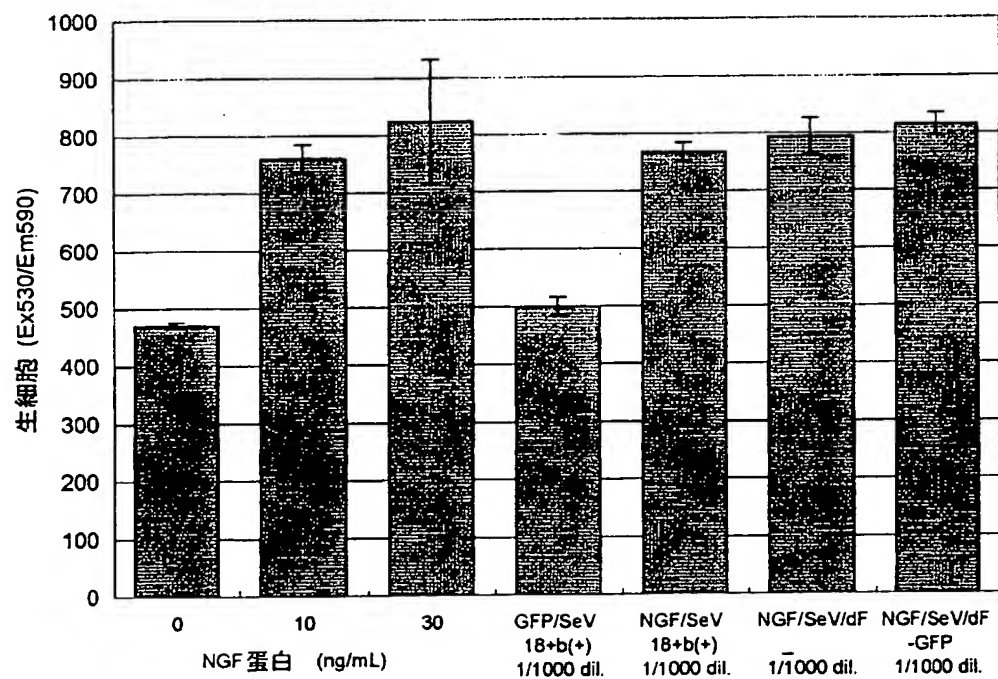
NGFプライマー (NGF-N~NGF-C)



1. マーカー
2. NGF/SeV/ΔF
3. NGF/SeV/ΔF-GFP
4. pSeV18<sup>+</sup>b(+)

56 / 62

図 5 6



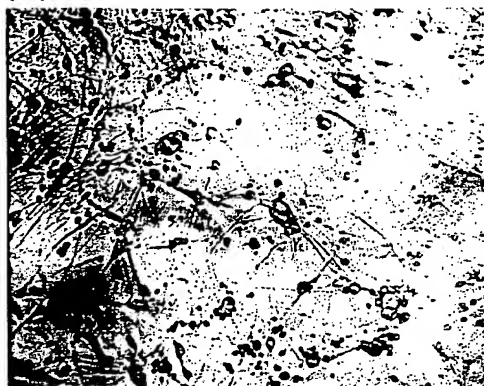
57 / 62

図 57

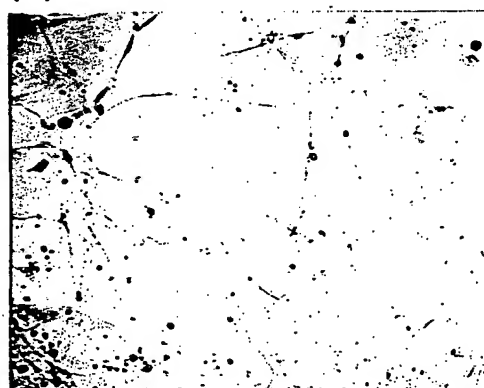
(A)



(B)



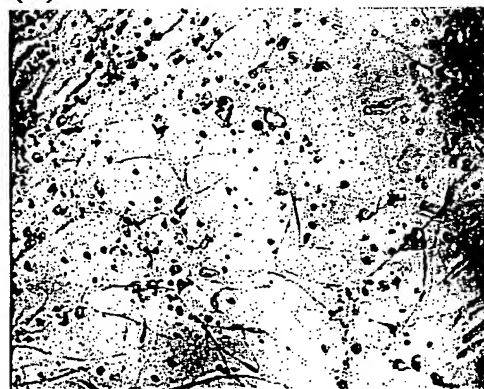
(C)



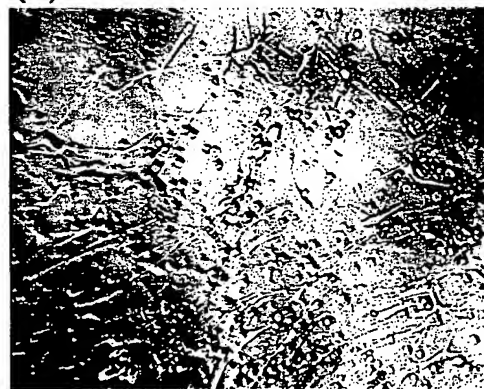
(D)



(E)

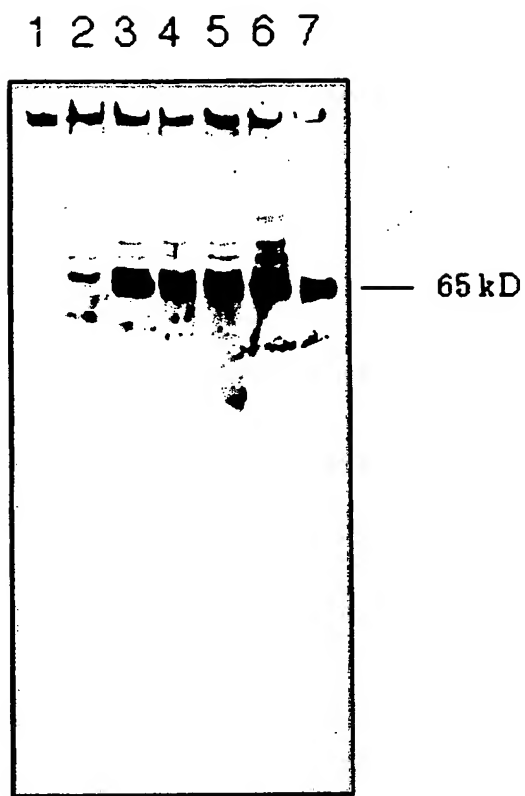


(F)



58 / 62

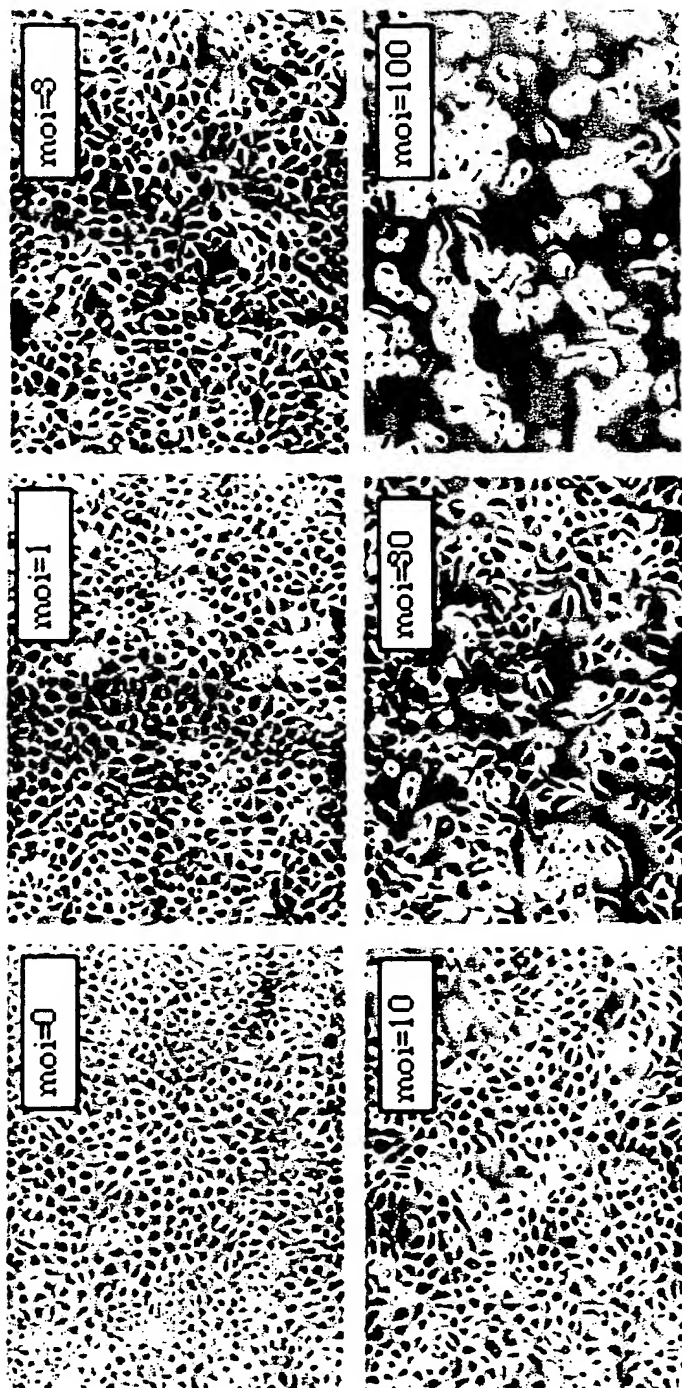
☒ 58



- 1: LLCMK2/Ad(moi=1)  
2: LLCMK2/F/Ad(moi=0)  
3: LLCMK2/F/Ad(moi=1)  
4: LLCMK2/F/Ad(moi=3)  
5: LLCMK2/F/Ad(moi=10)  
6: LLCMK2/F/Ad(moi=30)  
7: LLCMK2/F/Ad(moi=100)

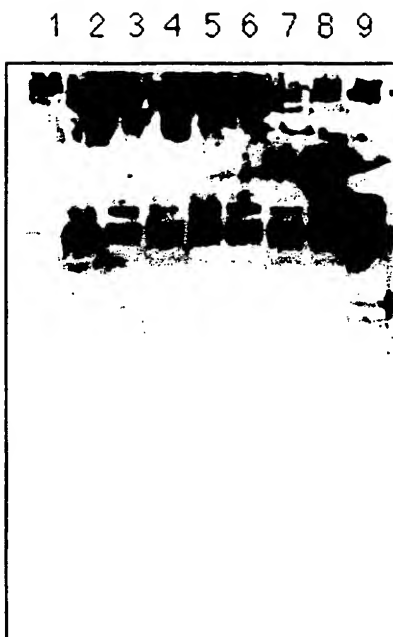
59 / 62

図 59



60/62

図 60



- 1 : LLC-MK2/Ad-Cre(Negative Control)
- 2 : LLC-MK2/F/Ad-Cre/P0
- 3 : LLC-MK2/F/Ad-Cre/P1
- 4 : LLC-MK2/F/Ad-Cre/P2
- 5 : LLC-MK2/F/Ad-Cre/P3
- 6 : LLC-MK2/F/Ad-Cre/P4
- 7 : LLC-MK2/F/Ad-Cre/P5
- 8 : LLC-MK2/F/Ad-Cre/P6
- 9 : LLC-MK2/F/Ad-Cre/P7

61/62

図 61

F6



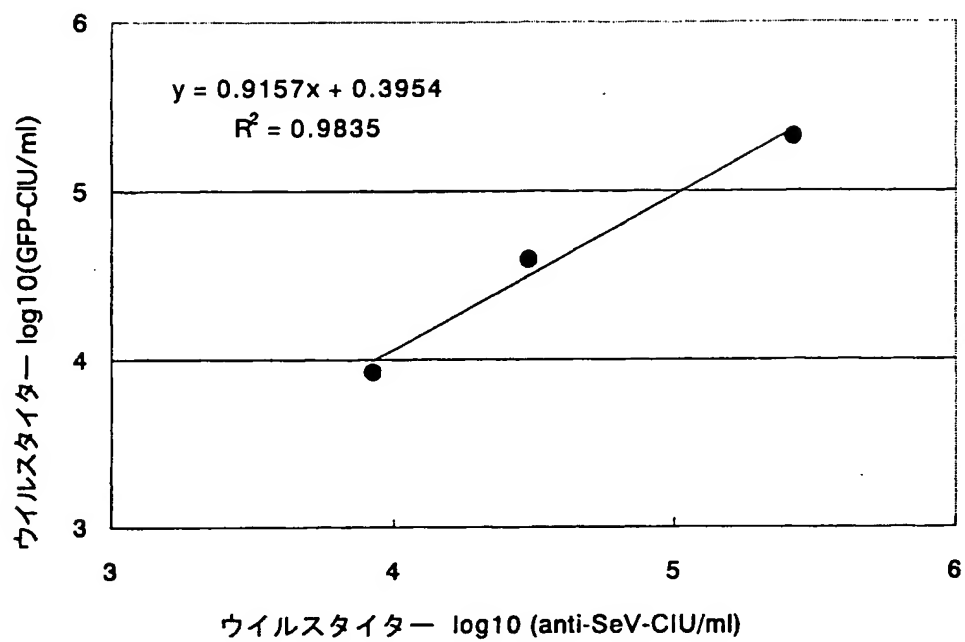
F20





62 / 62

図 6 2



1/25

SEQUENCE LISTING

<110> DNAVEC Research Inc.

<120> Paramyxovirus vectors deficient in envelope genes

<130> D3-103PCT

<140>

<141>

<150> JP 1999-200739

<151> 1999-05-18

<160> 52

<170> PatentIn Ver. 2.0

<210> 1

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Artificially  
Synthesized Sequence

2/25

&lt;400&gt; 1

atgcatgccg gcagatga

18

&lt;210&gt; 2

&lt;211&gt; 18

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Artificially  
Synthesized Primer Sequence

&lt;400&gt; 2

gttgagtact gcaagagc

18

&lt;210&gt; 3

&lt;211&gt; 42

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Artificially  
Synthesized Primer Sequence

&lt;400&gt; 3

3/25

tttgccggca tgcattgttc ccaaggggag agttttgcaa cc 42

<210> 4

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Artificially  
Synthesized Primer Sequence

<400> 4

atgcatgccg gcagatga 18

<210> 5

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Artificially  
Synthesized Primer Sequence

<400> 5

tgggtgaatg agagaatcag c 21

4/25

&lt;210&gt; 6

&lt;211&gt; 30

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Artificially  
Synthesized Primer Sequence

&lt;400&gt; 6

atgcatatgg tgatgcggtt ttggcagtac

30

&lt;210&gt; 7

&lt;211&gt; 30

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Artificially  
Synthesized Primer Sequence

&lt;400&gt; 7

tgccggctat tattacttgt acagctcgtc

30

&lt;210&gt; 8

&lt;211&gt; 21

5/25

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Artificially  
Synthesized Primer Sequence

&lt;400&gt; 8

atcagagacc tgcgacaatg c

21

&lt;210&gt; 9

&lt;211&gt; 21

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Artificially  
Synthesized Primer Sequence

&lt;400&gt; 9

aagtcgtgct gcttcattgtg g

21

&lt;210&gt; 10

&lt;211&gt; 25

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

6/25

&lt;220&gt;

<223> Description of Artificial Sequence: Artificially  
Synthesized Primer Sequence

&lt;400&gt; 10

acaaccacta cctgagcacc cagtc

25

&lt;210&gt; 11

&lt;211&gt; 21

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Artificially  
Synthesized Primer Sequence

&lt;400&gt; 11

gcctaacaca tccagagatc g

21

&lt;210&gt; 12

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

7/25

<223> Description of Artificial Sequence: Artificially  
Synthesized Primer Sequence

<400> 12

acattcatga gtcagctcgc

20

<210> 13

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Artificially  
Synthesized Primer Sequence

<400> 13

atcagagacc tgcgacaatg c

21

<210> 14

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Artificially  
Synthesized Primer Sequence



8/25

&lt;400&gt; 14

aagtcgtgct gcttcacgtg g

21

&lt;210&gt; 15

&lt;211&gt; 23

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Artificially  
Synthesized Primer Sequence

&lt;400&gt; 15

gaaaaactta gggataaagt ccc

23

&lt;210&gt; 16

&lt;211&gt; 19

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Artificially  
Synthesized Primer Sequence

&lt;400&gt; 16

9/25

gttatctccg ggatgggtgc

19

&lt;210&gt; 17

&lt;211&gt; 45

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 17

gcgcggccgc cgtacgggtgg caaccatgtc gtttactttg accaa

45

&lt;210&gt; 18

&lt;211&gt; 80

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 18

gcgcggccgc gatgaacttt caccctaagt ttttcttact acggcgtacg ctattacttc 60  
tgacaccaga ccaactggta 80

10/25

&lt;210&gt; 19

&lt;211&gt; 41

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 19

ccaccgacca caccagcgg ccgcgacagc cacggcttcg g

41

&lt;210&gt; 20

&lt;211&gt; 41

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 20

ccgaagccgt ggctgtcgcg gccgctgggt gtggtcggtg g

41

&lt;210&gt; 21

11/25

&lt;211&gt; 40

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 21

gaaatttcac ctaagcggcc gcaatggcag atatctatag

40

&lt;210&gt; 22

&lt;211&gt; 40

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 22

ctatagatat ctgccattgc ggccgcttag gtgaaatttc

40

&lt;210&gt; 23

&lt;211&gt; 43

&lt;212&gt; DNA

12/25

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 23

gggataaaagt cccttgcggc cgcttggttg caaaactctc ccc

43

&lt;210&gt; 24

&lt;211&gt; 43

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 24

ggggagagtt ttgcaaccaa gcggccgcaa gggactttat ccc

43

&lt;210&gt; 25

&lt;211&gt; 47

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

13/25

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 25

ggtcgcgcgg tacttttagcg gccgcctcaa acaagcacag atcatgg

47

&lt;210&gt; 26

&lt;211&gt; 47

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 26

ccatgatctg tgcttgtttg aggcggccgc taaagtaccg cgcgacc

47

&lt;210&gt; 27

&lt;211&gt; 44

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: artificially

14/25

synthesized sequence

&lt;400&gt; 27

cctgcccatac catgacctag cggccgcttc ccattcaccc tggg

44

&lt;210&gt; 28

&lt;211&gt; 44

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 28

cccagggtga atgggaagcg gccgctaggt catggatggg cagg

44

&lt;210&gt; 29

&lt;211&gt; 40

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

15/25

&lt;400&gt; 29

gcggcgcgcc atgctgctgc tgctgctgct gctgggcctg 40

&lt;210&gt; 30

&lt;211&gt; 40

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 30

gcggcgcgcc cttatcatgt ctgctcgaag cggccggcgcg 40

&lt;210&gt; 31

&lt;211&gt; 74

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 31

gcggccgcgt ttaaacggcg cgccatttaa atccgtagta agaaaaactt agggtgaaag 60



16/25

ttcatcgcg cgc

74

&lt;210&gt; 32

&lt;211&gt; 74

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 32

gcggccgcga tgaactttca ccctaagttt ttcttactac ggatttaa at ggcgcgccgt 60  
ttaa acgcgc cgc

74

&lt;210&gt; 33

&lt;211&gt; 48

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 33

acttgccgcc gccaaagttc agtaatgtcc atgtgttct acactctg

48

17/25

&lt;210&gt; 34

&lt;211&gt; 72

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 34

atccgcggcc gcgatgaact ttcaccctaa gtttttctta ctacggtcag cctcttcttg 60  
tagccttcct gc 72

&lt;210&gt; 35

&lt;211&gt; 40

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 35

ggagaagtct caacaccgtc cacccaagat aatcgatcag

40

18/25

&lt;210&gt; 36

&lt;211&gt; 40

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 36

ctgatcgatt atcttgggtg gacggtgtg agacttctcc

40

&lt;210&gt; 37

&lt;211&gt; 38

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 37

gtatatgtgt tcagttgagc ttgctgtcgg tctaaggc

38

&lt;210&gt; 38

&lt;211&gt; 38

19/25

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 38

gccttagacc gacagcaagc tcaactgaac acatatac

38

&lt;210&gt; 39

&lt;211&gt; 45

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 39

caatgaactc tctagagagg ctggagtcac taaagagtta cctgg

45

&lt;210&gt; 40

&lt;211&gt; 45

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

20/25

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 40

ccaggtaact ctttagtgac tccagcctct ctagagagtt cattg 45

&lt;210&gt; 41

&lt;211&gt; 52

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 41

gtgaaagttc atccaccgat cggctcactc gaggccacac ccaacccac cg 52

&lt;210&gt; 42

&lt;211&gt; 52

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

21/25

<223> Description of Artificial Sequence: artificially  
synthesized sequence

<400> 42

cggtaggggtt gggtgtggcc tcgagtgagc cgatcgggtg atgaactttc ac 52

<210> 43

<211> 47

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: artificially  
synthesized sequence

<400> 43

cttagggtga aagaaatttc agctagcacg ggcgaatggc agatatac 47

<210> 44

<211> 47

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: artificially  
synthesized sequence

22/25

&lt;400&gt; 44

gatatctgcc attgcgccgt gctagctgaa atttctttca ccctaag

47

&lt;210&gt; 45

&lt;211&gt; 47

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 45

cttagggata aagtccttg tgcgcgcttg gttgcaaaac tctcccc

47

&lt;210&gt; 46

&lt;211&gt; 47

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 46

23/25

ggggagagtt ttgcaaccaa gcgcgcacaa gggactttat ccctaag 47

<210> 47

<211> 47

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: artificially  
synthesized sequence

<400> 47

ggtcgcgcgg tacttttagtc gacacctcaa acaagcacag atcatgg 47

<210> 48

<211> 47

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: artificially  
synthesized sequence

<400> 48

ccatgatctg tgcttgtttg aggtgtcgac taaagtaccg cgcgacc 47



24/25

&lt;210&gt; 49

&lt;211&gt; 49

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 49

cccagggtga atgggaaggg ccggccaggt catggatggg caggagtcc 49

&lt;210&gt; 50

&lt;211&gt; 49

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 50

ggactcctgc ccattcatga cctggccggc ccttccatt caccctggg 49

&lt;210&gt; 51

&lt;211&gt; 72

25/25

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 51

ggccgcttaa ttaacggttt aaacgcgcgc caacagtgtt gataagaaaa acttaggtg 60  
aaagttcatc ac 72

&lt;210&gt; 52

&lt;211&gt; 72

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: artificially  
synthesized sequence

&lt;400&gt; 52

ggccgtgatg aactttcacc ctaagttttt cttatcaaca ctgttggcgc gcgtttaaac 60  
cgtaattaa gc 72

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/03195

## A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl.<sup>7</sup> C12N15/86, C12N15/45, C12N7/01 // C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl.<sup>7</sup> C12N15/00-90, C12N7/00-08, C12N5/00-28

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI (DIALOG), BIOSIS (DIALOG), MEDLINE (STN)

GenBank/EMBL/DDBJ/GeneSeq

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	J. Virol., Vol.74, No.14, (July 2000), pp.6564-6569, HAI-OU LI, et al., "A Cytoplasmic RNA Vector Derived from Nontransmissible Sendai Virus with Efficient Gene Transfer and Expression."	1-13
A	J. Virol., Vol.71, No.7 (1997), pp.5481-5486, M. Bitzer, et al., "Sendai Virus Efficiently Infects Cells via the Asialo-glycoprotein Receptor and Requires the Presence of Cleaved F <sub>0</sub> Precursor Proteins for This Alternative Route of Cell Entry."	1-13
PA	Biopolymers (Peptide Science), Vol.51, No.1 (June, 1999) pp.79-86, H. Kido, et al., "The Human Mucus Protease Inhibitor and Its Mutants are Novel Defensive Compounds Against Infection with Influenza A and Sendai Viruses."	1-13
A	Genes to Cells, Vol.2, No.7 (1997), pp.457-466, D. Yu, et al., "Sendai virus-based expression of HIV-1 gp120: reinforcement by the V(-) version."	1-13



Further documents are listed in the continuation of Box C.



See patent family annex.

\* Special categories of cited documents:  
 "A" document defining the general state of the art which is not considered to be of particular relevance  
 "E" earlier document but published on or after the international filing date  
 "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)  
 "O" document referring to an oral disclosure, use, exhibition or other means  
 "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  
 "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone  
 "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art  
 "&" document member of the same patent family

Date of the actual completion of the international search  
 15 August, 2000 (15.08.00)

Date of mailing of the international search report  
 19 September, 2000 (19.09.00)

Name and mailing address of the ISA/  
 Japanese Patent Office

Authorized officer

Facsimile No.

Telephone No.

## A. 発明の属する分野の分類 (国際特許分類 (IPC))

Int. Cl' C12N15/86, C12N15/45, C12N7/01 // C12N5/10

## B. 調査を行った分野

調査を行った最小限資料 (国際特許分類 (IPC))

Int. Cl' C12N15/00-90, C12N7/00-08, C12N5/00-28

最小限資料以外の資料で調査を行った分野に含まれるもの

国際調査で使用した電子データベース (データベースの名称、調査に使用した用語)

WPI (DIALOG), BIOSIS (DIALOG), MEDLINE (STN)  
GenBank/EMBL/DDBJ/GeneSeq

## C. 関連すると認められる文献

引用文献の カテゴリー*	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	関連する 請求の範囲の番号
T	J. Virol., Vol. 74, No. 14, (July 2000), p. 6564-6569, HAI-OU LI, et al. "A Cytoplasmic RNA Vector Derived from Nontransmissible Sendai Virus with Efficient Gene Transfer and Expression."	1-13
A	J. Virol., Vol. 71, No. 7 (1997) p. 5481-5486, M. Bitzer, et al. "Sendai Virus Efficiently Infects Cells via the Asialo- glycoprotein Receptor and Requires the Presence of Cleaved	1-13

☒ C欄の続きにも文献が列挙されている。☐ パテントファミリーに関する別紙を参照。

## \* 引用文献のカテゴリー

「A」特に関連のある文献ではなく、一般的技術水準を示すもの

「E」国際出願日前の出願または特許であるが、国際出願日以後に公表されたもの

「L」優先権主張に疑義を提起する文献又は他の文献の発行日若しくは他の特別な理由を確立するために引用する文献 (理由を付す)

「O」口頭による開示、使用、展示等に言及する文献

「P」国際出願日前で、かつ優先権の主張の基礎となる出願

の日の後に公表された文献

「T」国際出願日又は優先日後に公表された文献であって出願と矛盾するものではなく、発明の原理又は理論の理解のために引用するもの

「X」特に関連のある文献であって、当該文献のみで発明の新規性又は進歩性がないと考えられるもの

「Y」特に関連のある文献であって、当該文献と他の1以上の文献との、当業者にとって自明である組合せによって進歩性がないと考えられるもの

「&amp;」同一パテントファミリー文献

国際調査を完了した日

15.08.00

国際調査報告の発送日

19.09.00

国際調査機関の名称及びあて先

日本国特許庁 (ISA/JP)

郵便番号100-8915

東京都千代田区霞が関三丁目4番3号

特許庁審査官 (権限のある職員)

齊藤 真由美



4B

8931

電話番号 03-3581-1101 内線 3448

C (続き) . 関連すると認められる文献		
引用文献の カテゴリー*	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	関連する 請求の範囲の番号
	F <sub>0</sub> Precursor Proteins for This Alternative Route of Cell Entry."	
P A	Biopolymers (Peptide Science), Vol.51, No.1 (June,1999) p.79-86 , H.Kido, et al. " The Human Mucus Protease Inhibitor and Its Mutants are Novel Defensive Compounds Against Infection with Influenza A and Sendai Vieruses."	1 - 1 3
A	Genes to Cells, Vol.2, No.7 (1997) p.457-466, D.Yu, et al. " Sendai vierus-based expression of HIV-1 gp120: reinforcement by the V(-) version."	1 - 1 3

VERIFICATION OF TRANSLATION

I, Motonobu Namura

of 6th Fl., Kantetsu Tsukuba-Science-City Bldg. 1-1-1, Oroshi-machi, Tsuchiura, Ibaraki,  
JAPAN

declare as follows:

1. That I am well acquainted with both the English and Japanese languages, and
2. That the attached document is a true and correct translation of a certified copy of the following application, which was made by me to the best of my knowledge and belief.

- (a) International Patent Application No. PCT/JP00/03195  
Entitled: "Envelope Gene-Deficient Paramyxovirus Vector"  
Filed on May 18, 2000

July 1, 2005  
(Date)

Motonobu Namura  
(Signature of Translator)  
Motonobu Namura

## DESCRIPTION

## ENVELOPE GENE-DEFICIENT PARAMYXOVIRUS VECTOR

5 Technical Field

The present invention relates to an envelope gene-deficient viral vector of Paramyxoviridae.

Background Art

10 In many clinical approaches of gene therapy until now, viral vectors from retroviruses, adenoviruses, and adeno-associated viruses have been used. These gene therapy vectors have limitations in gene introducing efficiency and persistent expression, and also have cell toxicity, and immunogenicity, which are crucial problems  
15 when it comes to the medical application of these vectors (Lamb, R.A. & Kolakofsky, D., Paramyxoviridae: the viruses and their replication. in Fields Virology, 3rd edn, (Edited by B. N. Fields, D. M. Knipe & P. P. Howley) pp.1177-1204 (Philadelphia, Lippincott-Raven (1996))). Novel vectors based on lentiviruses and HSV have been proposed as  
20 countermeasures, and extensive research is also being carried out to improve existing vectors. However, all of these vectors exist in the form of DNA within the nucleus throughout the life cycle. Therefore, it is difficult to fully overcome concerns of safety related to random interactions with the patient's chromosomes.

25 Recent rapid progress of reverse genetics technologies is making it possible to develop vectors based on RNA viruses, the development of which has been long delayed. Recombinant RNA virus vectors show high gene introduction efficiency and expression capability, and thus show a very high potentiality as vectors for  
30 gene therapy (Roberts, A. & Rose, J. K., Virology 247, 1-6 (1998); Rose, J., Proc. Natl. Acad. Sci. USA 94, 14998-15000 (1996); Palese, P. et al., Proc. Natl. Acad. Sci. USA 93, 11354-11358 (1996)). However, practically usable paramyxovirus vectors derived from deficient type genome of attenuated viruses have not been reported yet.

35 Paramyxovirus vectors having negative-strand RNA as the genome have several characteristics significantly different from

retroviruses, DNA viruses or positive-strand RNA virus vectors. Genomes or antigenomes of negative-strand RNA viruses do not directly function as mRNA, so they cannot initiate the synthesis of viral proteins and genome replication. Both RNA genome and antigenome of these viruses always exist in the form of a ribonucleoprotein complex (RNP), so they hardly cause problems caused by antisense strands, such as interfering with the assembly of genome to RNP due to mRNA hybridizing with naked genomic RNA, as in the case of positive strand RNA viruses. These viruses comprise their own RNA polymerases, performing the transcription of viral mRNAs or replication of viral genomes using RNP complex as the template. Worthy of mentioning is that negative-strand RNA (nsRNA) viruses proliferate only in the cytoplasm of host cells, causing no integration thereof into chromosomes, because they do not go through a DNA phase. Furthermore, no homologous recombination among RNAs has been recognized. These properties are considered to contribute a great deal to the stability and safety of negative-strand RNA viruses as gene expressing vectors.

Among negative-strand RNA viruses, the present inventors have been focusing their attention on the Sendai virus (SeV). Sendai virus is a non-segmented type negative-strand RNA virus belonging to the genus Paramyxovirus, and is a type of murine parainfluenza virus. The virus attaches to the host cell membrane via envelope glycoproteins, the hemagglutinin-neuraminidase (HN) and fusion protein (F), causes membrane fusion, and efficiently releases its own RNA polymerase and the RNA genome, which exists as a ribonucleoprotein (RNP) complex, into the cytoplasm, and carries out mRNA transcription of the virus and genome replication at the site (Bitzer, M. et al., J. Virol. 71(7):5481-5486, 1997). The viral envelope protein F is synthesized as an inactive precursor protein ( $F_0$ ), then divided into F1 and F2 by proteolytic cleavage with trypsin-like protease such as triptase clara (Kido, H. et al., Biopolymers (Peptide Science) 51(1): 79-86, 1999), and thus becomes an active form protein to cause membrane fusion. This virus has been said to be non-pathogenic towards humans. However, wild-type SeV has been said highly cytopathic in cell culture (D. Garcin, G. Taylor, K. Tanebayashi, R. Compans and D. Kolakofsky, Virology 243, 340-353



(1998)). Therefore, we focused research on Z strain of SeV, an attenuated laboratory strain of Sendai virus, which has been isolated, and which only induces mild pneumonia in rodents, the natural hosts. This strain has been widely used as a research model for molecular level studies of the transcription-replication mechanism of paramyxoviruses and used for preparing hybridomas. In addition to the high safety mentioned above, the virus shows a high production titer of  $10^{9-11}$  pfu/ml in cell lines or chicken eggs. In one recently successful recovery system of negative-strand RNA virus vector from cDNA, especially high reconstitution efficiency has been seen in the case of Sendai virus. The capability of recombinant wild type viruses introduced with exogenous genes, to efficiently and stably express introduced exogenous genes is gaining wide attention.

Thus, negative-strand RNA viruses have many advantages as gene introducing vectors. However, to apply for gene therapy, the development of highly safe vectors that do not release infectious particles when infected to cells is desired. For that purpose, a technique that mass produces viruses deficient in wild type virus production capability is necessary. However, development of an applicable vector based on an envelope gene-deficient genome has not yet been successful.

#### Disclosure of the Invention

The aim of present invention is to provide a paramyxovirus vector deficient in an envelope gene.

To construct a paramyxovirus vector suitable for gene therapy, which completely lacks a propagation capability, the present inventors deleted F gene of SeV from the genome to establish a method to recover infectious virus particles in cells expressing F protein of Sendai virus, using cDNA in which GFP gene is introduced as a reporter. Through this F gene-deficient virus vector, a gene is introduced into rat neuronal cells in primary cultures, primitive mouse blood stem cells, human normal cells, and various other types of cells with a high efficiency, and a high expression was seen. Furthermore, high expression was obtained when administrated into rat brain *in vivo*. The F gene-deficient SeV vector expresses a gene

relatively persistently and strongly in the infected cells without producing secondary infectious virus particles, and does not propagate within adjacent cells. Thus, the usefulness of the vector for gene therapy was suggested.

5 Furthermore, the present inventors produced a SeV vector cDNA deficient in both F gene and HN gene, to establish a method to recover infectious virus particles in a cell line expressing F protein and HN protein of Sendai virus. In addition, by introducing the SeV vector cDNA into F-expressing cells, the present inventors succeeded in  
10 constructing SeV vector deficient in the HN protein.

Thus, the present invention establishes an applicable novel envelope gene-deficient vector system based on a negative-strand RNA virus for the first time. The success in the recovery of infectious deficient virus particles from F gene-deficient, or FHN  
15 gene-deficient genomic cDNA using helper cells pave the way for research and development of novel vectors for gene therapy taking advantage of the remarkable characteristics of Sendai virus.

The deficient Sendai virus vector of the present invention has an extremely high gene-introducing efficiency towards various cell  
20 types and an enormous capability of expressing an exogenous gene. Furthermore, it expresses persistently in infected cells and does not release infectious virus particles, proving that it is a highly safe vector completely without virus-propagating capability.

From cells infected with F or HN gene-deficient Sendai virus  
25 vectors, non-infectious virus-like particles (VLPs, also called F-less virions or HN-less virions) are released (see Examples 7, Figure 21, and Stricker, R. and Roux, L., J. Gen. Virol. 72: 1703-1707 (1991)). Because M protein plays central role in release of virus-like particles, virus vectors deficient in M gene lose their  
30 particle forming capability or have the capability extremely reduced, and can diffuse vectors to adjacent cells through cell fusion (WO00/09700; Mebatsion, T. et al., J. Virol. 73: 242-250 (1990); Cathomen, T. et al. EMBO J. 17: 3899-3908 (1998)). The present inventors have first established helper cells which are capable of  
35 expressing M protein stably. Using the helper cells stably expressing M proteins, the present inventors produced M

gene-deficient Sendai virus vectors comprising M proteins as envelopes. Infectious M gene-deficient virus vectors are prepared in high titer (released from the producer cells into culturing medium at more than  $10^7$  Cell Infectious Units (CIU)/ml), and it has been revealed from Western blot analysis and measurement of HA activity that few VLPs are released from cells infected with M gene-deficient virus vectors. Moreover, the present inventors constructed a Sendai virus cDNA deficient in both F gene and M gene and reconstituted vectors using helper cells expressing F and M proteins. In particular, virus vectors deficient in M gene in addition to F or HN gene are extremely useful as vectors for gene therapy because reinfection of virus from cells into which the vectors are introduced and cell damage and immunity induction due to secondary release are not induced. In fact, the present inventors have succeeded in producing infectious virus particles deficient in both M and F genes in the culture supernatant of virus producing cells at the titer of  $10^8$  CIU/ml or more at the maximum for the first time. The virus thus obtained lost almost all the secondary virus particle forming capability. Furthermore, it was confirmed that cytotoxicity of the viral vector deficient in both M and F genes remarkably decreased compared to that of vectors deficient in either one of these two genes. This viral vector has been demonstrated to be capable of efficient gene transfer into nerve cells *in vivo* and *in vitro* and therefore would be used as a gene transfer vector having infectivity towards many types of cells including nondividing cells.

The stability of genome is pointed out as a problem when using RNA viruses. Heterologous gene expression by SeV vector showed hardly any base mutations after continual multiple passages, showing that it expresses the inserted heterologous gene stably for a long period (Yu, D. et al. *Genes cells* 2, 457-466 (1997)). Vectors based on negative-strand RNA virus replicons have several advantageous characteristics such as genome stability or flexibility of the size of the gene introduced or packaging, for they do not have the capsid structural protein, when compared to vectors based on replicons of Semliki forest virus, an already successful positive-strand RNA virus, or those of Sindbis virus. At least 4 kbp of exogenous DNA can be

inserted into the wild type Sendai virus vector, and a much longer one can be inserted into the deficient vector. By inserting, between genes, a unit comprising a transcription initiation sequence, two or more kinds of genes can be expressed simultaneously. Persistent  
5 expression is expected in the vector based on replicon of Sendai virus since theoretically, except for human peripheral monocytes (F. Tropea et al., Exp. Cell. Res. 218(1), 63-70 (1995)), multicopied RNPs replicated in the cytoplasm are distributed into daughter cells when cell division occurs. Actually, this has been demonstrated in an in  
10 vitro study in a certain kind of blood cells. Furthermore, since the present inventors have confirmed that the Sendai virus vector is introduced with a high efficiency into blood cells, especially granulocytic cells, and also that it is introduced into c-kit positive cells, the vector is thought to be a very highly applicable vector  
15 with a very extensive tissue application range.

Thus, the present invention relates to envelope gene-deficient Sendai virus vector, more specifically to:

- (1) A paramyxovirus vector comprising a complex comprising (a) a paramyxovirus-derived negative-strand single-stranded RNA modified  
20 not to express at least one envelope protein of paramyxoviruses, and (b) proteins that bind to said negative-strand single-stranded RNA.
- (2) The vector according to (1), wherein the negative-strand single-stranded RNA expresses NP protein, P protein, and L protein, and is modified not to express F, HN, or M protein, or any combination  
25 thereof.
- (3) The vector according to (1), comprising at least one of the envelope proteins whose expression was suppressed in the modified negative-strand single-stranded RNA.
- (4) A vector according to (1), comprising VSV-G protein.
- 30 (5) A vector according to (1), wherein the negative-strand single-stranded RNA is derived from Sendai virus.
- (6) A vector according to (1), wherein the negative-strand single-stranded RNA further encodes an exogenous gene.
- (7) A DNA encoding negative-strand single-stranded RNA comprised in  
35 a vector according to any one of (1) to (6), or the complementary strand thereof.

(8) A method for producing a vector according to (1), comprising the following steps of:

(a) expressing vector DNA encoding a paramyxovirus-derived negative-strand single-stranded RNA modified not to express at least one envelope protein of paramyxoviruses, or the complementary strand, by introducing into cells expressing at least one envelope protein, (b) culturing said cells, and, (c) recovering the virus particles from the culture supernatant.

(9) A method for producing a vector according to (1), comprising the steps of,

(a) introducing, a complex comprising a paramyxovirus-derived negative-strand single-stranded RNA modified not to express at least one envelope protein of paramyxoviruses, and a protein binding to said negative-strand single-stranded RNA, into cells expressing at least one envelope protein, (b) culturing said cells, and, (c) recovering virus particles from the culture supernatant.

(10) The method according to (8) or (9), wherein the cell culture in (b) is a co-culture with cells expressing at least one envelope protein.

(11) The method according to (8) or (9), wherein cells expressing at least one envelope protein are overlaid to said cells in cell culture in (b).

(12) The method of (8) or (9), wherein the cell culture is carried out at 35°C or less.

(13) A method according to (8) or (9), wherein at least one envelope protein expressed by the cells is identical to at least one envelope protein whose expression is suppressed in the negative-strand single-stranded RNA described above.

(14) A method according to (8) or (9), wherein at least one envelope protein expressed by the cells is VSV-G protein.

In the present invention, the term "vector" indicates virus particles in which nucleic acid molecules for expressing exogenous gene in hosts are packaged.

The term "recombinant" used herein means a compound or a

composition generated by mediating a recombinant polynucleotide. A recombinant polynucleotide means a polynucleotide in which nucleotide residues are linked not naturally, namely, a polynucleotide in which nucleotide residues are not arranged in a manner found in nature.

5 Herein, a "recombinant" paramyxovirus vector means a paramyxovirus vector constructed by genetic engineering or a paramyxovirus vector obtained by amplifying it. Recombinant paramyxovirus vectors can be generated, for example, by reconstituting recombinant paramyxovirus cDNAs.

10 "NP, P, M, F, HN and L genes" of viruses belonging to the family Paramyxoviridae refer to genes encoding nucleocapsid, phospho, matrix, fusion, hemagglutinin-neuraminidase and large proteins, respectively. Respective genes of viruses belonging to subfamilies of the family Paramyxoviridae are represented in general as follows.

15 NP gene is generally described also as the "N gene".

Genus	N	P/C/V	M	F	HN	-	L
Respirovirus							
Genus	N	P/V	M	F	HN	(SH)	L
Rubulavirus							
Genus	N	P/C/V	M	F	H	-	L
Morbillivirus							

Database accession numbers for nucleotide sequences of genes of the Sendai virus classified into Respirovirus of the family Paramyxoviridae are, M29343, M30202, M30203, M30204, M51331, M55565, M69046 and X17218 for NP gene, M30202, M30203, M30204, M55565, M69046, 20 X00583, X17007 and X17008 for P gene, D11446, K02742, M30202, M30203, M30204, M69046, U31956, X00584 and X53056 for M gene, D00152, D11446, D17334, D17335, M30202, M30203, M30204, M69046, X00152 and X02131 for F gene, D26475, M12397, M30202, M30203, M30204, M69046, X00586, X02808 and X56131 for HN gene, and D00053, M30202, M30203, M30204, 25 M69040, X00587 and X58886 for L gene.

Herein, the term "particle forming capability" refers to the capability of a viral vector to release infectious or noninfectious virus particles (called virus-like particles) in cells infected with 30 said viral vector (referred to as the secondary release). Herein,

that "particle forming capability is reduced or suppressed" means that particle forming capability is significantly reduced. In addition, the reduction of particle forming capability includes the complete elimination of particle forming capability.

5       The reduction of particle forming capability refers to, for example, a statistically significant reduction thereof (e.g. level of significance: 5% or less). Statistical examination can be performed, for example, by Student's t-test, Mann-Whitney's U-test or the like. The level of particle forming capability decreases to  
10   1/2 or less, more preferably 1/5, 1/10, 1/30, 1/50, 1/100, 1/300 and 1/500 or less of the wild type virus.

      The elimination of particle forming capability means that the level of VLP is below the detection limits. In such cases, VLP is  $10^3$ /ml or less, preferably  $10^2$ /ml or less, more preferably  $10^1$ /ml or  
15   less. The elimination of particle forming capability can be determined by means of a functional assay. For example, its elimination can be confirmed when no detectable infectivity is observed in cells transfected with a sample that may contain VLP. Moreover, virus particles can be identified with a direct observation  
20   tool such as an electron microscope, or detected and quantified from nucleic acid or protein contained in virus as an indicator. For example, genomic nucleic acid contained in virus particles may be detected and quantified by the usual method for detecting nucleic acid such as PCR. Alternatively, virus particles having a foreign  
25   gene can be quantified by transfecting cells with them and detecting the expression of said gene in the cells. Noninfectious virus particles (e.g. VLP) can be quantified by introducing these particles into cells in combination with a transfection reagent and detecting the expression of the foreign gene. The transfection can be carried  
30   out, for example, by using lipofection reagents. The following is an example of the transfection using DOSPER Liposomal Transfection Reagent (Roche, Basel, Switzerland; Cat No. 1811169). DOSPER (12.5  $\mu$ l) is mixed with 100  $\mu$ l of a solution with or without VLP, and the mixture is allowed to stand still at room temperature for 10 minutes.  
35   The mixed solution is used to transfect cells which have been cultured to be confluent on a 6-well plate with shaking every 15 minutes. After

2 days, the presence or absence of VLP can be determined by detecting the presence or absence of infected cells. Infective viruses can be quantified by normal CIU assay or hemagglutination activity (HA) assay (Kato, A. et al., 1996, *Genes Cells* 1: 569-579; Yonemitsu, Y. & Kaneda, Y., Hemagglutinating virus of Japan-liposome-mediated gene delivery to vascular cells. Ed. by Baker AH. *Molecular Biology of Vascular Diseases. Method in Molecular Medicine*: Humana Press: pp. 295-306, 1999).

The term "gene" used herein means a genetic substance, which includes nucleic acids such as RNA, DNA, etc. In general, a gene may or may not encode a protein. For example, a gene may be that encoding a functional RNA such as ribozyme, antisense RNA, etc. A gene may have a naturally derived or artificially designed sequence. In addition, herein, a "DNA" includes a single-stranded DNA and a double-stranded DNA.

The present invention relates to envelope gene-deficient paramyxovirus vectors. The virus vector comprises paramyxovirus-derived negative-strand single-stranded RNA modified not to express at least one envelope protein. Paramyxovirus generally comprises a complex of RNA and protein (ribonucleoprotein; RNP) in the envelope. The RNA comprised in RNP is negative-strand (negative-strand) single-stranded RNA, which is the genome of paramyxovirus. The protein binds to the RNA to form the complex. Namely, a paramyxovirus vector according to this invention comprises a complex comprising (a) a paramyxovirus-derived negative-strand single-stranded RNA modified so as not to express at least one of the envelope proteins of paramyxoviruses and (b) proteins binding to said negative-strand single-stranded RNA. Proteins binding to a negative-strand single-stranded RNA refer to proteins binding directly and/or indirectly to the negative-strand single-stranded RNA to form an RNP complex with the negative-strand single-stranded RNA. In general, negative-strand single-stranded RNA (genomic RNA) of paramyxovirus is bound to NP, P and L proteins. RNA contained in this RNP serves as the template for transcription and replication of RNA (Lamb, R. A., and D. Kolakofsky, 1996, *Paramyxoviridae: The*



viruses and their replication, pp. 1177-1204. In *Fields Virology*, 3<sup>rd</sup> edn. Fields, B. N., D. M. Knipe, and P. M. Howley et al. (ed.), Raven Press, New York, N. Y.). Complexes of this invention include those comprising negative-strand single-stranded RNAs originating in paramyxovirus and proteins also originating in paramyxovirus which bind to the RNAs. Vectors of this invention comprise RNP comprising, for example, negative-strand single-stranded RNA of paramyxoviruses to which these proteins (NP, P and L proteins) are bound. In general, RNP complexes of paramyxovirus are capable of autonomously self-replicating in host cells. Thus, vectors transferred to cells intracellularly proliferate RNP to increase the copy number of the gene (RNA contained in complex), thereby leading to a high level expression of a foreign gene from RNP carrying the foreign gene. Vectors of this invention are preferably those capable of replicating RNA contained in complexes (RNP) in transfected cells.

Herein, paramyxovirus means a virus belonging to the family Paramyxoviridae or a derivative thereof. In addition to the Sendai virus, the Paramyxoviridae virus for which the present invention can be applied is, for instance, measles virus, simian parainfluenza virus (SV5), and human parainfluenza virus 3, but is not limited to thereto. Other examples of paramyxoviruses include Newcastle disease virus, Mumps virus, Respiratory syncytial (RS) virus, rinderpest virus, distemper virus, human parainfluenza virus type 1 and 2, etc. The paramyxovirus of the present invention is preferably a virus belonging to the genus *Paramyxovirus* or a derivative thereof. Examples of viruses of the genus *Paramyxovirus* to which the present invention can be applied include human parainfluenza virus type 1 (HPIV-1), human parainfluenza virus type 3 (HPIV-3), bovine parainfluenza virus type 3 (BPIV-3), Sendai virus (also called mouse parainfluenza virus type 1), simian parainfluenza virus type 10 (SPIV-10), etc. The paramyxovirus of the present invention is most preferably Sendai virus. These viruses may be derived from natural strains, wild-type strains, mutant strains, laboratory-passaged strains, artificially constructed strains, etc. Incomplete viruses such as the DI particle (J. Virol. 68, 8413-8417 (1994)), synthesized oligonucleotides, and so on, can also be utilized as material for producing the virus vector

of the present invention.

The paramyxovirus vector of the present invention is a particle separated from cells and a particle having infectivity and not having disseminative capability. The term "infectivity" used  
5 herein means capability of a vector to transfer a gene contained in the vector, to a cell by adhering to the cell. The term "not having disseminative capability" means that particles having infectivity are not released from cells infected with the virus vector.

Negative-strand single-stranded RNAs contained in viral  
10 vectors are modified, typically, so as to express NP, P and L proteins and so as not to express F, HN, or M protein, or any combination thereof. Preferably, the negative-strand single-stranded RNAs contained in the viral vectors of the present invention are modified so as not to express at least F and/or HN proteins. The present invention  
15 particularly relates to a viral vector having a negative-strand single-stranded RNA that has been modified so as not to express two or more proteins selected from F, HN, and M proteins. More specifically, this invention provides a viral vector having a negative-strand single-stranded RNA that has been modified so as not  
20 to express at least F and HN proteins, F and M proteins, or M and HN proteins. A viral vector that does not express F protein has the advantage of having no cytotoxicity such as syncytium formation. A viral vector that does not express HN protein has the advantage of not causing hemagglutination. A viral vector that does not express  
25 M protein has the advantage of not releasing VLP. Viral vectors prepared by deleting any combination of genes encoding these viral proteins have the combination of the respective advantages.

Furthermore, the present invention provides a method for attenuating cytotoxicity caused by gene transfer, the method  
30 comprising the step of transfecting cells with a viral vector deficient in genes encoding the envelope proteins (for example, F, HN or M gene, or combinations thereof) described herein. The present invention also provides a method for suppressing release of virus-like particles (VLPs) from cells transfected with a viral vector upon gene  
35 transfer, the method comprises the step of transfecting cells with the above-described viral vector. Cytotoxicity can be measured, for

example, by quantifying the level of LDH release as described in Examples. Release of virus-like particles (VLPs) can be detected, for example, by measuring HA activity as described in Examples. Alternatively, VLP contained in the extracellular fluid of the transfected cells can be quantified by collecting the extracellular fluid, transfecting other cells with the fluid and measuring the expression level of the gene contained in VLP. It is preferable that cytotoxicity is attenuated and VLP release is suppressed to, for example, a statistically significant level (e.g. the significance level of 5% or less) compared to a viral vector without the above-described gene deletion. Statistical examination can be performed, for example, by Student's t-test, Mann-Whitney's U-test, etc. The cytotoxicity is attenuated and VLP release is suppressed to 90% or less, preferably to 80% or less, more preferably to 70% or less, still more preferably 60% or less, still further preferably to 1/2 or less, 1/3 or less, 1/5 or less or 1/8 or less, compared to the wild-type virus.

The term "not expressing a protein" used herein includes a case where the protein is substantially not expressed. A protein is not expressed from the genomic RNA in a virus vector by making a gene encoding the protein deficient. "Deficiency" of a gene means that any functional gene product (which is a protein if the gene encodes the protein) of the gene is substantially not expressed. The deficiency of a gene of interest includes a case where null phenotype is indicated for the gene. The deficiency of a gene includes that the gene is deleted; that the gene is not transcribed due to mutation of a transcription initiation sequence and so on; that no functional protein is produced due to frameshift, codon mutation, or the like; that activity of the expressed protein is substantially lost [or decreased very much (for example, 1/10 or less)] due to amino acid mutation and so on; that translation into a protein does not occur [or is decreased very much (for example, 1/10 or less)]; and so on.

In the case of Sendai virus (SeV), the genome of the natural virus is approximately 15,000 nucleotides in size, and the negative-strand comprises six genes encoding NP (nucleocapsid), P

(phospho), M (matrix), F (fusion), HN (hemagglutinin-neuraminidase) and L (large) proteins lined in a row following the 3'-short leader region, and a short 5'-trailer region on the other end. In this invention, this genome can be modified so as not to express envelope proteins and/or matrix proteins by designing a genome deficient in any of F, HN and M genes, or any combination thereof. Deficiency in either F gene or HN gene, or both is preferred. In addition, it is preferable that M gene is deficient. Since these proteins are unnecessary for the formation of RNP, RNPs which are components of the vectors of this invention can be manufactured by transcribing this genomic RNA (either positive or negative-strand) in the presence of NP, P and L proteins. RNP formation can be performed, for example, in LLC-MK2 cells, or the like. NP, P and L proteins can be supplied by introducing to cells expression vectors carrying the respective genes for these proteins (cf. Examples). Each gene may be also incorporated into chromosomes of host cells. NP, P and L genes to be expressed for the formation of RNP need not be completely identical to those genes encoded in the genome of the vector. That is, amino acid sequences of proteins encoded by these genes may not be identical to those of proteins encoded by RNP genome, as long as they can bind to the genomic RNA and are capable of replicating RNP in cells, and these genes may be induced with mutations or replaced with homologous genes from other viruses. Once an RNP is formed, NP, P and L genes are expressed from this RNP to autonomously replicate RNP in the cells and produce viral vectors in the presence of envelope proteins. In addition, the virus gene arrangement on the genome of the paramyxovirus of the present invention may be modified from that on the wild-type or mutant virus genome. For example, the short leader region of rSev<sup>GP42</sup> (D. Garcin et al, Virology, 243, 340-353 (1998)) could be replaced with its counterpart genome sequence of SeV.

If an envelope protein is expressed in cells when a vector is reconstituted, this envelope protein will be incorporated into the vector, enabling the production of viral vectors with infectivity due to the envelope protein. Such a vector, once infected to cells, cannot produce viruses comprising an envelope protein as the initial virus can, because it does not have the envelope gene, though it can

propagate RNP within the cells. Such a vector is very useful in fields such as gene therapy where exceptionally high safety is required.

5 Viral vectors with equivalent infection capability as the wild type virus can be produced by expressing the envelope proteins whose expression is suppressed in modified negative-strand single-stranded RNA, namely envelope genes deficient in the genome, at the time of virus reconstitution. Expressing a portion of envelope genes deficient in the genome is also conceivable. For example, when F protein alone is expressed against the genome deficient in both F and HN gene, a virus vector with F protein as envelope is produced. 10 The virus with only F protein, but without HN protein, can be used as a vector that infects specifically to hepatocytes, mediated by asialoglycoprotein receptor (ASG-R). Thus, paramyxovirus vectors comprising at least one envelope protein whose expression is 15 suppressed in modified negative-strand single-stranded RNA are included in the present invention.

In addition, it is also possible to reconstitute the vector of the present invention by using envelope proteins other than the above-described proteins whose expression was suppressed by modifying 20 negative-strand single-stranded RNA. For example, virus vectors having desired envelope proteins other than those encoded by the genome of the virus which is the base of the vectors can be produced by expressing the envelope proteins in cells when the virus is reconstituted. There is no particular limitation on the type of such 25 envelope proteins. One example of other viral envelope proteins is the G protein (VSV-G) of vesicular stomatitis virus (VSV). The paramyxovirus vector of the present invention includes pseudo-type viral vectors comprising envelope protein derived from a virus different to the virus from which the genome is derived, such as VSV-G 30 protein, and the like.

Viral vectors of this invention can be usually prepared by (a) introducing a vector DNA encoding paramyxovirus-derived negative-strand single-stranded RNA that has been modified so as not to express at least one of the viral envelope proteins of 35 paramyxoviruses, or a complementary strand of said RNA, into cells (helper cells) expressing one or more envelope proteins, and allowing

the vector DNA to be expressed, and (b) culturing the cells to recover viral particles from the culture supernatant. By coexpressing NP, P and L proteins at the time of vector DNA expression, RNPs are formed and a virus having envelope proteins is constructed. Envelope proteins expressed in cells may be constitutively or, at the time of viral reconstitution, inducibly expressed in the cells.

By culturing the cells at low temperature in the step (b), the efficiency of virus vector production can be significantly increased. Therefore, it is preferable that the cells are cultured in the step (b) at low temperature, namely 35°C or less, more preferably 34°C or less, even more preferably 33°C or less, and most preferably 32°C or less.

Vector DNA to be expressed in helper cells encodes negative-strand single-stranded RNA contained in vectors of this invention (negative-strand) or complementary strand thereof (positive-strand). For example, DNA encoding negative-strand single-stranded RNA or complementary strand thereof is linked downstream of T7 promoter to be transcribed to RNA by T7 RNA polymerase. Desired promoters can be used except those including the recognition sequence of T7 polymerase. Alternatively, RNA transcribed *in vitro* may be transfected into helper cells. Vector DNAs may be cloned into plasmids to amplify in *E. coli*. Although the strand to be transcribed inside cells may be either positive or negative-strand, it is well known that virus reconstitution efficiency is preferably improved by arranging so as to transcribe the positive strand (A. Kato, Y. Sakai, T. Shioda, T. Kondo, M. Nakanishi, Y. Nagai, *Genes to Cells*, 1, 569-579 (1996)).

As helper cells, cells expressing envelope protein are used. As described above, helper cells are not limited to cells expressing all proteins of envelope genes deficient in the virus vector, for instance, for F, HN gene-deficient Sendai virus vector DNA, cells expressing F protein alone can be used as helper cells. In addition, cells expressing envelope protein different to the protein encoded by the envelope gene deficient in the virus vector may also be used. For example, as described above, an envelope protein that is not the envelope protein of paramyxovirus such as VSV-G protein can also be

used as an envelope protein.

Helper cells that express the envelope proteins can be obtained by transfecting cells with an expression vector carrying the genes encoding these proteins and selecting the cells into which the genes have been stably incorporated. It is preferable that the envelope proteins can be expressed by way of induction. Examples of the cell include, for example, simian kidney-derived cell line LLC-MK2. The high level expression of the envelope proteins in helper cells is important for harvesting the virus with a high titer. For that purpose, it is preferable to perform, for example, the above-described transfection and cell selection at least twice or more. For example, cells are transfected with an envelope protein expression plasmid carrying a drug-resistance marker gene and the cells into which the envelope protein gene has been introduced are selected using the drug. Then, the selected cells are transfected with an envelope protein expression plasmid carrying a different drug-resistance marker gene and the second selection is made using this different drug resistance marker. This selection method enables to select cells capable of expressing the envelope protein at a higher level than those selected by the first transfection. Such envelope protein expressing helper cells which have been constructed via twice or more transfections can be preferably used. Such twice or more transfections are important for preparation of helper cells expressing M protein in particular. Furthermore, helper cells simultaneously expressing two or more envelope proteins, for example, M and F proteins are preferably prepared by twice or more transfections of cells with not only the M protein expression plasmid but also the F protein expression plasmid so as to enhance the induction level of F protein expression.

The helper cells thus obtained can be used to reconstitute the vector according to the present invention. For example, a viral vector can be reconstituted by transfecting a plasmid expressing a recombinant Sendai virus vector genome deficient in one or more envelope genes into host cells together with a vector expressing one or more envelope proteins; and NP, P and L protein expression vectors. Alternatively, RNP complex can be manufactured using, for example,

host cells incorporated with F gene into chromosomes thereof. Amino acid sequences of these proteins supplied from outside the viral genome need not be identical to those deriving from the virus. As long as these proteins are equally active to or more active than natural type proteins in the ability of transferring nucleic acids into cells, genes encoding these proteins may be modified by inserting some mutations or replacing with homologous genes from other viruses. Since, in general, many envelope proteins, solely or in combination, show cytotoxicity (C. M. Horvath et al., J. Virology, 66, 4564-4569 (1992); X. Hu et al., J. Virology, 66, 1528-1534 (1992); R. Lamb, Virology, 197, 1-11 (1993); A. Moscona and R. W. Peluso, J. Virology, 65, 2773-2777 (1991)), and therefore, they may be arranged to be expressed only when the vector is reconstituted under the control of an inducible promoter or the expression can be induced at the time of reconstitution using other mechanism that can regulate the expression (cf. Examples).

Once RNP or virus comprising RNP is formed, virus vectors of this invention can be amplified by introducing this RNP or virus again into the aforementioned helper cells and culturing them. This process comprises the steps of (a) introducing a complex comprising negative-strand single-stranded RNA derived from paramyxovirus modified not to express at least one envelope protein of paramyxoviruses, and proteins that binds to said negative-strand single-stranded RNA to cells expressing envelope proteins, and (b) culturing the cells and recovering virus particles from the culture supernatant.

RNP may be introduced to cells as a complex formed together with, for example, lipofectamine and a polycationic liposome. Specifically, a variety of transfection reagents can be utilized. Examples thereof are DOTMA (Boehringer), Superfect (QIAGEN #301305), DOTAP, DOPE, DOSPER (Boehringer #1811169), etc. Chloroquine may be added to prevent RNP from decomposition in endosomes (Calos, M. P., 1983, Proc. Natl. Acad. Sci. USA 80: 3015).

Once a viral vector is thus constructed in host cells, it can be further amplified by coculturing these cells with cells expressing envelope proteins. As described in Example 12, a preferable example



is the method of overlaying cells expressing envelope proteins over virus producing cells.

As an envelope protein, besides a viral envelope protein, for example, a chimeric protein comprising, in its extracellular region, a polypeptide derived from an adhesion molecule, ligand, receptor protein, and such that can adhere to specific cells, and in its intracellular region, polypeptides derived from virus envelope can be used. Hereby, vectors targeted to specific tissues can be produced. Viral vectors of this invention, for example, may comprise a viral gene contained in the vector that has been modified to reduce the antigenicity or enhance the RNA transcription and replication efficiency. Specifically, for example, as for paramyxovirus, it is possible to modify at least one of the NP, P/C, and L genes, which are genes of replication factors, to enhance the function of transcription or replication. In addition, the HN protein is a structural protein having both hemagglutinin activity and neuraminidase activity, and it is possible to enhance the virus stability in blood, for example, by weakening the former activity and to regulate infectivity, for example, by altering the latter activity. It is also possible to regulate the fusion ability by altering the F protein, which is implicated in membrane fusion. Furthermore, it is possible to generate a virus vector that is engineered to have weak antigenicity against these proteins through analyzing the antigen presenting epitopes and such of possible antigenic molecules on the cell surface such as the F protein and HN protein.

In addition, paramyxovirus whose accessory gene is deficient can be used as the paramyxovirus of the present invention. For example, by knocking out V gene, one of the accessory genes of SeV, pathogenicity of SeV to hosts such as mice markedly decreases without damages to the expression and replication of genes in cultured cells (Kato, A. et al., 1997, J. Virol. 71: 7266-7272; Kato, A. et al., 1997). Such attenuated vectors are particularly preferable as virus vectors for *in vivo* or *ex vivo* gene transfer.

Viral vectors of this invention may include RNA encoding a foreign gene in their negative-strand single-stranded RNA. Any gene

desired to be expressed in target cells may be used as the foreign gene. For example, when gene therapy is intended, a gene for treating an objective disease is inserted into the viral vector DNA. In the case where a foreign gene is inserted into the viral vector DNA, for example, Sendai viral vector DNA, it is preferable, to insert a sequence comprising a nucleotide number of a multiple of six between the transcription termination sequence (E) and transcription initiation sequence (S), etc. (Calain, P. and Roux, L., Journal of Virology, Vol. 67, No. 8, 1993, p.4822-4830). Foreign gene may be inserted before or after each of the virus genes (NP, P, M, F, HN and L genes) (cf. Examples). E-I-S sequence (transcription termination sequence-intervening sequence-transcription initiation sequence) or portion thereof is appropriately inserted before or after a foreign gene and a unit of E-I-S sequence is located between each gene so as not to interfere with the expression of genes before or after the foreign gene. Expression level of the inserted foreign gene can be regulated by the type of transcription initiation sequence added upstream of the foreign gene, as well as the site of gene insertion and nucleotide sequences before and after the gene. For example, in Sendai virus, the nearer the insertion site is to the 3'-end of negative-strand RNA (in the gene arrangement on the wild type viral genome, the nearer to NP gene), the higher the expression level of the inserted gene is. To secure a high expression level of a foreign gene, it is preferable to insert the foreign gene into upstream region, namely at the 3'-side in negative-strand genome such as upstream of NP gene (the 3'-side in negative-strand) or between NP and P genes. Conversely, the nearer the insertion position is to the 5'-end of negative-strand RNA (in the gene arrangement on the wild type viral genome, the nearer to L gene), the lower the expression level of the inserted gene is. To suppress the expression of a foreign gene to a low level, the foreign gene is inserted, for example, to the far most 5'-side of the negative-strand, that is, downstream of L gene in the wild type viral genome (the 5'-side adjacent to L gene in negative-strand) or upstream of L gene (the 3'-side adjacent to L gene in negative-strand). Thus, the insertion position of a foreign gene can be properly adjusted so as to obtain a desired expression

level of the gene or so as to optimize the combination of it and the virus protein-encoding genes before and after it. For instance, if the overexpression of a gene introduced by inoculating a high titer virus vector may cause toxicity, it is possible not only to limit the titer of the virus to be inoculated, but also to reduce the expression level from individual virus vectors, for example, by designing the insertion position on the vector as closely to the 5'-terminus of the negative-strand as possible, or replacing the transcription initiation sequence with one having lower efficiency so as to obtain an appropriate therapeutic effect.

Because, in general, it is advantageous to obtain high expression of an foreign gene as long as cytotoxicity is not raised, it is preferable to ligate the foreign gene with a highly efficient transcription initiation sequence and to insert the gene into the vicinity of the 3'-terminus of the negative-strand genome. Examples of preferable vectors include a vector in which the foreign gene is located at the 3'-side of any virus protein genes of paramyxovirus in the negative-strand genome of paramyxovirus vector. For example, a vector in which the foreign gene is inserted upstream (at the 3'-side of the negative-strand) of N gene is preferable. Alternatively, the foreign gene may be inserted immediately downstream of N gene.

To facilitate the insertion of a foreign gene, a cloning site may be designed at the inserting position in the vector DNA encoding the genome. The cloning site can be arranged to be, for example, the recognition sequence for restriction enzymes. Foreign gene fragments can be inserted into the restriction enzyme site in the vector DNA encoding the genome. Cloning site may be arranged to be a so-called multi-cloning site comprising a plurality of restriction enzyme recognition sequences. Vectors of this invention may harbor other foreign genes at the sites other than those described above.

Recombinant Sendai virus vectors comprising a foreign gene can be constructed as follows according to, for example, the description in "Hasan, M. K. et al., J. Gen. Virol. 78: 2813-2820, 1997", "Kato, A. et al., 1997, EMBO J. 16: 578-587" and "Yu, D. et al., 1997, Genes Cells 2: 457-466".

First, a DNA sample comprising the cDNA nucleotide sequence of

a desired foreign gene is prepared. It is preferable that the DNA sample can be electrophoretically identified as a single plasmid at concentrations of 25 ng/ $\mu$ l or more. Below, a case where a foreign gene is inserted to DNA encoding viral genome utilizing NotI site will be described as an example. When NotI recognition site is included in the objective cDNA nucleotide sequence, it is preferable to delete the NotI site beforehand by modifying the nucleotide sequence using site-specific mutagenesis and such method so as not to alter the amino acid sequence encoded by the cDNA. From this DNA sample, the desired gene fragment is amplified and recovered by PCR. To have NotI sites on the both ends of amplified DNA fragment and further add a copy of transcription termination sequence (E), intervening sequence (I) and transcription initiation sequence (S) (EIS sequence) of Sendai virus to one end, a forward side synthetic DNA sequence (sense strand) and reverse side synthetic DNA sequence (antisense strand) are prepared as a pair of primers containing NotI restriction enzyme cleavage site sequence, transcription termination sequence (E), intervening sequence (I), transcription initiation sequence (S) and a partial sequence of the objective gene.

For example, to secure cleavage by NotI, the forward side synthetic DNA sequence is arranged in a form in which any two or more nucleotides (preferably 4 nucleotides excluding GCG and GCC, sequences originating in NotI recognition site, more preferably ACTT) are selected on the 5'-side of the synthetic DNA, NotI recognition site "gcggccgc" is added to its 3'-side, and to the 3'-side thereof, any desired 9 nucleotides or nucleotides of 9 plus a multiple of 6 nucleotides are added as the spacer sequence, and to the 3'-side thereof, about 25 nucleotide-equivalent ORF including the initiation codon ATG of the desired cDNA is added. It is preferable to select about 25 nucleotides from the desired cDNA as the forward side synthetic DNA sequence so as to have G or C as the final nucleotide on its 3'-end.

In the reverse side synthetic DNA sequence, any two or more nucleotides (preferably 4 nucleotides excluding GCG and GCC, sequences originating in the NotI recognition site, more preferably ACTT) are selected from the 5'-side of the synthetic DNA, NotI

recognition site "gcggccgc" is added to its 3'-side, and to its further 3'-side, an oligo DNA is added as the insertion fragment to adjust the length. This oligo DNA is designed so that the total nucleotide number including the NotI recognition site "gcggccgc", complementary sequence of cDNA and EIS nucleotide sequence of Sendai virus genome originating in the virus described below becomes a multiple of six (so-called "rule of six"; Kolakofski, D. et al., J. Virol. 72: 891-899, 1998; Calain, P. and Roux, L., J. Virol. 67: 4822-4830, 1993). Further to the 3'-side of inserted fragment, a sequence complementary to S sequence of Sendai virus, preferably 5'-CTTTCACCCT-3' (SEQ ID NO: 63), I sequence, preferably 5'-AAG-3', and a sequence complementary to E sequence, preferably 5'-TTTTTCTTACTACGG-3' (SEQ ID NO: 64), is added, and further to the 3'-side thereof, about 25 nucleotide-equivalent complementary sequence counted in the reverse direction from the termination codon of the desired cDNA sequence the length of which is adjusted to have G or C as the final nucleotide, is selected and added as the 3'-end of the reverse side synthetic DNA.

PCR can be done according to the usual method with, for example, ExTaq polymerase (Takara Shuzo). Preferably, PCR is performed using Vent polymerase (NEB), and desired fragments thus amplified are digested with NotI, then inserted to NotI site of the plasmid vector pBluescript. Nucleotide sequences of PCR products thus obtained are confirmed with a sequencer to select a plasmid having the right sequence. The inserted fragment is excised from the plasmid using NotI, and cloned to the NotI site of the plasmid carrying the genomic cDNA deficient in one or more envelope genes. Alternatively, it is also possible to obtain the recombinant Sendai virus cDNA by directly inserting the fragment to the NotI site without the mediation of the plasmid vector pBluescript.

It is also possible to transcribe a viral vector DNA of the present invention in test tubes or cells, reconstitute RNP with viral L, P and NP proteins, and produce the virus vector comprising this RNP. Reconstitution of virus from the viral vector DNA can be carried out according to methods known in the art using cells expressing envelope proteins (WO97/16539 and 97/16538: Durbin, A. P. et al.,

1997, Virology 235: 323-332; Whelan, S. P. et al., 1995, Proc. Natl. Acad. Sci. USA 92: 8388-8392; Schnell, M. J. et al., 1994, EMBO J. 13: 4195-4203; Radecke, F. et al., 1995, EMBO J. 14: 5773-5784; Lawson, N. D. et al., Proc. Natl. Acad. Sci. USA 92: 4477-4481; Garcin, D. et al., 1995, EMBO J. 14: 6087-6094; Kato, A. et al., 1996, Genes Cells 1: 569-579; Baron, M. D. and Barrett, T., 1997, J. Virol. 71: 1265-1271; Bridgen, A. and Elliott, R. M., 1996, Proc. Natl. Acad. Sci. USA 93: 15400-15404). These methods enable reconstituting, from DNA, desired paramyxovirus vectors including the parainfluenza virus, vesicular stomatitis virus, rabies virus, measles virus, rinderpest virus, Sendai virus vectors, etc. When a viral vector DNA is made deficient in F, HN and/or M genes, infectious virus particles are not formed with such a defective vector by itself. However, it is possible to form infectious virus particles by separately transferring these deficient genes, genes encoding other viral envelope proteins, and such, to host cells and expressing them therein.

Methods for transferring viral vector DNA into cells include the following: 1) the method of preparing DNA precipitates that can be taken up by objective cells; 2) the method of preparing a DNA comprising complex which is suitable for being taken up by objective cells and which is also not very cytotoxic and has a positive charge, and 3) the method of instantaneously boring on the objective cellular membrane pores wide enough to allow DNA molecules to pass through by electric pulse.

In Method 2), a variety of transfection reagents can be utilized, examples being DOTMA (Boehringer), Superfect (QIAGEN #301305), DOTAP, DOPE, DOSPER (Boehringer #1811169), etc. An example of Method 1) is a transfection method using calcium phosphate, in which DNA that entered cells are incorporated into phagosomes, and a sufficient amount is incorporated into the nuclei as well (Graham, F. L. and Van Der Eb, J., 1973, Virology 52: 456; Wigler, M. and Silverstein, S., 1977, Cell 11: 223). Chen and Okayama have investigated the optimization of the transfer technique, reporting that optimal DNA precipitates can be obtained under the conditions where 1) cells are incubated with DNA in an atmosphere of 2 to 4% CO<sub>2</sub> at 35°C for 15 to

24 h, 2) cyclic DNA with a higher precipitate-forming activity than linear DNA is used, and 3) DNA concentration in the precipitate mixture is 20 to 30  $\mu\text{g/ml}$  (Chen, C. and Okayama, H., 1987, Mol. Cell. Biol. 7: 2745). Method 2) is suitable for a transient transfection. An old method is known in the art in which a DEAE-dextran (Sigma #D-9885, M.W.  $5 \times 10^5$ ) mixture is prepared in a desired DNA concentration ratio to perform the transfection. Since most of the complexes are decomposed inside endosomes, chloroquine may be added to enhance transfection effects (Calos, M. P., 1983, Proc. Natl. Acad. Sci. USA 80: 3015). Method 3) is referred to as electroporation, and is more versatile compared to methods 1) and 2) because it doesn't have cell selectivity. Method 3) is said to be efficient under optimal conditions for pulse electric current duration, pulse shape, electric field potency (gap between electrodes, voltage), conductivity of buffers, DNA concentration, and cell density.

Among the above-described three categories, transfection reagents (method 2)) are suitable in the case where nucleic acids or RNPs are introduced into cells for vector reconstitution in this invention, because method 2) is easily operable, and facilitates the examining of many test samples using a large amount of cells. Preferably, Superfect Transfection Reagent (QIAGEN, Cat. No. 301305) or DOSPER Liposomal Transfection Reagent (Boehringer Mannheim, Cat. No. 1811169) is used, but the transfection reagents are not limited thereto.

Specifically, the reconstitution of the viral vector from cDNA can be performed as follows.

Simian kidney-derived LLC-MK2 cells are cultured in 24-well to 6-well plastic culture plates or 100 mm diameter culture dish and such using a minimum essential medium (MEM) containing 10% fetal calf serum (FCS) and antibiotics (100 units/ml penicillin G and 100  $\mu\text{g/ml}$  streptomycin) to 70 to 80% confluency, and infected, for example, with recombinant vaccinia virus vTF7-3 expressing T7 polymerase at 2 PFU/cell. This virus has been inactivated by a UV irradiation treatment for 20 min in the presence of 1  $\mu\text{g/ml}$  psoralen (Fuerst, T. R. et al., Proc. Natl. Acad. Sci. USA 83: 8122-8126, 1986; Kato, A. et al., Genes Cells 1: 569-579, 1996). Amount of psoralen added

and UV irradiation time can be appropriately adjusted. One hour after the infection, the cells are transfected with 2 to 60  $\mu\text{g}$ , more preferably 3 to 5  $\mu\text{g}$ , of the above-described recombinant Sendai virus cDNA by the lipofection method and such using plasmids (24 to 0.5  $\mu\text{g}$  of pGEM-N, 12 to 0.25  $\mu\text{g}$  of pGEM-P and 24 to 0.5  $\mu\text{g}$  of pGEM-L, more preferably 1  $\mu\text{g}$  of pGEM-N, 0.5  $\mu\text{g}$  of pGEM-P and 1  $\mu\text{g}$  of pGEM-L) (Kato, A. et al., Genes Cells 1: 569-579, 1996) expressing trans-acting viral proteins required for the production of full-length Sendai viral genome together with Superfect (QIAGEN). The transfected cells are cultured in a serum-free MEM containing 100  $\mu\text{g}/\text{ml}$  each of rifampicin (Sigma) and cytosine arabinoside (AraC) if desired, more preferably only containing 40  $\mu\text{g}/\text{ml}$  of cytosine arabinoside (AraC) (Sigma), and concentrations of reagents are set at optima so as to minimize cytotoxicity due to the vaccinia virus and maximize the recovery rate of the virus (Kato, A. et al., 1996, Genes Cells 1, 569-579). After culturing for about 48 to 72 h following the transfection, the cells are recovered, disrupted by repeating three cycles of freezing and thawing, transfected to LLC-MK2 cells expressing envelope proteins, and cultured. After culturing the cells for 3 to 7 days, the culture solution is collected. Alternatively, infectious virus vectors can be obtained more efficiently by transfecting LLC-MK2 cells already expressing envelope proteins with plasmids expressing NP, L and P proteins, or transfecting together with an envelope-expressing plasmid. When plasmids expressing F and HN proteins is used for the envelope protein expression, the quantity ratios of plasmids expressing the genomic RNA, N, P, L, F and HN proteins may be set, for example, at 6:2:1:2:2:2 (in terms of the copy number of transcriptional unit). When a plasmid expressing M protein is co-transfected, it can be used in the same amount as that of the F protein expression plasmid. Conditions of transfection are not limited thereto, however, and can be appropriately optimized. Viral vectors can be amplified by culturing these cells overlaid on LLC-MK2 cells expressing envelope proteins (cf. Examples). Virus titer contained in the culture supernatant can be determined by measuring the hemagglutination activity (HA), which can be assayed by "endo-point dilution method" (Kato, A. et al., 1996, Genes Cells 1, 569-579).



Virus stock thus obtained can be stored at  $-80^{\circ}\text{C}$ .

According to the method of the present invention, it is possible to release the viral vector of this invention into the extracellular fluid (culture supernatant) of the virus producing cells at the titer, for example, of  $1 \times 10^5$  CIU/ml or more, preferably  $1 \times 10^6$  CIU/ml or more,  $5 \times 10^6$  CIU/ml or more,  $1 \times 10^7$  CIU/ml or more,  $5 \times 10^7$  CIU/ml or more,  $1 \times 10^8$  CIU/ml or more, and  $5 \times 10^8$  CIU/ml or more. Furthermore, the present invention relates to a mammalian cell containing genes encoding envelope proteins of paramyxovirus integrated into its chromosome, which cell is capable of producing an infectious paramyxoviral vector deficient in said genes. This cell is capable of releasing said vector into the extracellular fluid at the titer of, for example,  $1 \times 10^5$  CIU/ml or more, preferably  $1 \times 10^6$  CIU/ml or more,  $5 \times 10^6$  CIU/ml or more,  $1 \times 10^7$  CIU/ml or more,  $5 \times 10^7$  CIU/ml or more,  $1 \times 10^8$  CIU/ml or more, and  $5 \times 10^8$  CIU/ml or more. Virus production can be carried out by the method described herein. Preferably, the cell maintains the genes encoding the envelope proteins in such a manner as to inducibly express the proteins. Inducible expression refers to the expression induced by a specific stimulus or under specific conditions, and such an expression system can be constituted using, for example, an inductive promoter, Cre/lox P, and such. The cell may maintain two or more genes encoding paramyxovirus envelope proteins. For example, a combination of the genes encoding F and HN proteins, F and M proteins, or HN and M proteins, are integrated into chromosome of the cell.

A preferred embodiment for reconstituting viral vectors of the present invention is a method comprising the steps of: (a) transcribing the vector DNA encoding the negative strand RNA or the complementary strand thereof (positive strand) deficient in genes encoding envelope proteins derived from the negative-strand RNA virus in cells expressing viral proteins that are required for formation of infectious viral particles (that is, NP, P and L proteins as well as products of envelope protein genes deficient in the above-described genome), and (b) co-culturing said cells with cells that contains the envelope protein genes deficient in the above-described genome

incorporated in their chromosomes and are capable of expressing said proteins. The virus can be harvested from the culture supernatant of these cells. Preferably, the method further comprises, after the step (b), the steps of: (c) preparing cell extracts from the culture medium of (b), (d) introducing said extracts into cells containing envelope protein genes deficient in the above-described genome integrated into their chromosomes and culturing the cells, and (e) harvesting viral particles from the culture supernatant. The step (d), in particular, is preferably performed under the aforementioned lower temperature conditions. Virus particles thus obtained can be amplified by allowing them to infect the envelope protein expressing cells (preferably at low temperature). Specifically, the virus can be reconstituted as described in Examples. Envelope protein genes are not limited to those deficient in the genome, but any desired envelope protein genes capable of conferring infectivity on virus, such as VSV-G, may be used.

Recombinant Sendai virus vectors of this invention can be appropriately diluted, for example, with physiological saline and phosphate-buffered physiological saline (PBS) to prepare a composition. When recombinant Sendai virus vectors of this invention are proliferated in chicken eggs and such, the composition can include chorioallantoic fluid. Compositions comprising recombinant Sendai virus vectors of this invention may contain physiologically acceptable media such as deionized water, 5% dextrose aqueous solution, and so on, and, furthermore, other stabilizers and antibiotics may also be contained.

The type of host cells used for virus reconstitution is not particularly limited, so long as viral vector can be reconstituted therein. For example, in the reconstitution of Sendai virus vector or RNP complex, culture cells such as simian kidney-derived CV-1 cells and LLC-MK2 cells, hamster kidney-derived BHK cells, human-derived cells, and so on can be used. Infectious virus particles having the envelope can be also obtained by expressing appropriate envelope proteins in these cells. To obtain Sendai virus vector in a large quantity, the vector can be amplified, for example, by infecting virus

vector obtained from the above-described host cells into embryonated chicken eggs together with vectors expressing envelope genes. Alternatively, viral vectors can be produced using transgenic chicken eggs in which envelope protein genes have been introduced. Methods  
5 for manufacturing viral fluid using chicken eggs have been already developed (Nakanishi, et al. (eds.), 1993, "Shinkei-kagaku Kenkyu-no Sentan-gijutu Protocol III (High Technology Protocol III of Neuroscience Research), Molecular Neurocyte Physiology, Koseisha, Osaka, pp.153-172). Specifically, for example, fertilized eggs are  
10 placed in an incubator and incubated for 9 to 12 days at 37 to 38°C to grow embryos. Sendai virus vector is inoculated together with vectors expressing envelope proteins into chorioallantoic cavity of eggs, and cultured for several days to proliferate the virus. Conditions such as culture duration may be varied depending on the  
15 type of recombinant Sendai virus used. Subsequently, chorioallantoic fluid comprising the virus is recovered. Separation and purification of Sendai virus vector can be performed according to the standard methods (Tashiro, M., "Virus Experiment Protocols", Nagai and Ishihama (eds.), Medicalview, pp. 68-73 (1995)).

20 As a vector to express envelope proteins, viral vectors themselves of this invention may be used. For example, when two types of vectors in which a different envelope gene is deficient in the viral genome are transferred to the same cell, the envelope protein deficient in one vector is supplied by the expression of the other  
25 vector to complement each other, thereby leading to the formation of infectious virus particles and completion of replication cycle to amplify the viral vectors. That is, when two or more types of vectors are inoculated to cells in combinations so as to complement each other's envelope proteins, mixtures of virus vectors deficient  
30 in respective envelope proteins can be produced on a large scale and at a low cost. Mixed viruses thus produced are useful for the production of vaccines and such. Due to the deficiency of envelope genes, these viruses have a smaller genome size compared to the complete virus, so they can harbor a long foreign gene. Also, since  
35 these originally non-infectious viruses are extracellularly diluted, and it's difficult to retain their coinfection, they become sterile,

which is advantageous in managing their release to the environment.

Recovered paramyxovirus can be purified so as to be substantially pure. Purification can be performed by known purification and separation methods including filtration, centrifugation, column chromatographic purification, and such or by combination thereof. The term "substantially pure" used herein means that virus occupies the main ratio as a component of the sample in which the virus exists. Typically, substantially pure virus vectors can be detected by confirming that the ratio of the virus-derived proteins to the total proteins including in the sample occupies 50% or more, preferably 70% or more, more preferably 80% or more, and even more preferably 90% or more. Specifically, paramyxovirus can be purified, for example, by a method in which cellulose sulfate ester or crosslinked polysaccharide sulfate ester is used (Examined  
10 Published Japanese Patent Application (JP-B) No. Sho 62-30752; JP-B Sho 62-33879; JP-B Sho 62-30753), a method in which adsorption to fucose sulfate-containing polysaccharide and/or a decomposition product thereof is used (WO97/32010), etc.

Gene therapy is enabled by administering viral vectors when the viral vectors are prepared by using a therapeutic gene as the foreign gene. In the application of viral vectors of this invention to gene therapy, it is possible to express a foreign gene with which treatment effects are expected or an endogenous gene the supply of which is insufficient in the patient's body, by either direct or indirect (ex  
20 vivo) administration of the vector. There is no particular limitation on the type of foreign gene, and in addition to nucleic acids encoding proteins, they may be nucleic acids encoding no proteins, such as an antisense or ribozyme. In addition, when genes encoding antigens of bacteria or viruses involved in infectious  
25 diseases are used as foreign genes, immunity can be induced in animals by administering these genes to the animals. That is, vectors carrying these genes can be used as vaccines.

When using as vaccines, viral vectors of the present invention may be applicable for, for example, cancers, infectious diseases and  
30 other general disorders. For example, as cancer a treatment, it is possible to express genes with therapeutic effects on tumor cells

or antigen presenting cells (APC) such as dendritic cells (DCs) by using the vectors of the invention. Examples of such genes are those encoding the tumor antigen Muc-1 or Muc-1 like mutin tandem repeat peptide (US Patent No. 5,744,144), melanoma gp100 antigen, etc. Such treatments with genes have been widely applied to cancers in the mammary gland, colon, pancreas, prostate, lung, etc. Combination with cytokines to enhance adjuvant effects is also effective in gene therapy. Examples of such genes are i) single-chain IL-12 in combination with IL-2 (Proc. Natl. Acad. Sci. USA 96 (15): 8591-8596, 5 ii) interferon- $\gamma$  in combination with IL-2 (US Patent No. 5,798,100), 10 iii) granulocyte colony-stimulating factor (GM-CSF) used alone, and iv) GM-CSF aiming at the treatment of brain tumor in combination with IL-4 (J. Neurosurgery, 90 (6), 1115-1124 (1999)), etc.

Examples of genes used for the treatment of infectious diseases are those encoding the envelope protein of the virulent strain H5N1 15 type of influenza virus, the envelope chimera protein of Japanese encephalitis virus (Vaccine, vol. 17, No. 15-16, 1869-1882 (1999)), the HIV gag or SIV gag protein of AIDS virus (J. Immunology (2000), vol. 164, 4968-4978), the HIV envelope protein, which is incorporated 20 as a oral vaccine encapsulated in polylactate- glycol copolymer microparticles for administration (Kaneko, H. et al., Virology 267, 8-16 (2000)), the B subunit (CTB) of cholera toxin (Arakawa, T. et al., Nature Biotechnology (1998) 16 (10): 934-8; Arakawa, T. et al., Nature Biotechnology (1998) 16 (3): 292-297), the glycoprotein of 25 rabies virus (Lodmell, D. L. et al., 1998, Nature Medicine 4 (8): 949-52), and the capsid protein L1 of human papilloma virus 6 causing cervical cancer (J. Med. Virol., 60, 200-204 (2000)).

Gene therapy may also be applied to general disorders. For example, in the case of diabetes, the expression of insulin peptide 30 fragment by inoculation of plasmid DNA encoding the peptide has been performed in type I diabetes model animals (Coon, B. et al., J. Clin. Invest., 1999, 104 (2): 189-94).

Compositions containing paramyxovirus vectors are useful as reagents and pharmaceuticals. Dose of the vectors may vary depending 35 on a disease, body weight, age, sex, symptom, administration purpose, form of a composition to be inoculated, administration method, gene

to be introduced, and so on, but it can be properly determined by one skilled in the art. It is preferable to inoculate, with pharmaceutically acceptable carriers, the vectors whose concentration is within the range of preferably about  $10^5$  pfu/ml to about  $10^{11}$  pfu/ml, more preferably about  $10^7$  pfu/ml to about  $10^9$  pfu/ml, and most preferably about  $1 \times 10^8$  pfu/ml to about  $5 \times 10^8$  pfu/ml. The dosage for humans is preferably in the range of  $2 \times 10^5$  CIU to  $2 \times 10^{10}$  CIU and may be administered once or several times within the range of clinically acceptable side effects. Number of administration per day is similarly determined within the above-described range. The dosage to animals other than humans can be determined by, for example, multiplying the aforementioned human dosage value by the body weight ratio or volume ratio (e.g. values on the average) of targeted administration parts between animals of interest and humans. The subject of inoculation of the compositions containing paramyxovirus vectors includes all mammals such as humans, monkeys, mice, rats, rabbits, sheep, bovines, dogs, etc.

#### Brief Description of the Drawings

Figure 1 is a photograph showing an analytical result of the expression of F protein via a Cre-loxP-inducible expression system by Western blotting. It shows the result of detecting proteins on a transfer membrane cross-reacting to the anti-SeV-F antibody by chemiluminescence method.

Figure 2 indicates a diagram showing an analytical result of cell-surface display of F protein the expression of which was induced by the Cre-loxP system. It shows results of flow cytometry analysis for LLC-MK2/F7 with the anti-SeV-F antibody.

Figure 3 indicates a photograph showing the result confirming cleavage of the expressed F protein by trypsin using Western blotting.

Figure 4 indicates photographs showing the result confirming cell-surface expression of HN in an experiment of cell-surface adsorption onto erythrocytes.

Figure 5 indicates photographs showing the result obtained by an attempt to harvest the deficient viruses by using cells expressing the deficient protein. It was revealed that the expression of F

protein by the helper cell line was stopped rapidly by the vaccinia viruses used in the reconstitution of F-deficient SeV.

1. LLC-MK2 and CV-1 represent cell lysates from the respective cell types alone.
- 5 2. LLC-MK2/F+ad and CV-1/F+ad represent cell lysates from the respective cells that have been subjected to the induction of expression and to which adenovirus AxCANCre has been added.
3. LLC-MK2/F-ad and CV-1/F-ad represent cell lysates from the respective cell lines in which the F gene but no adenovirus AxCANCre  
10 has been introduced.
4. LLC-MK2/F+ad 3rd represents a cell lysate from cells in which the expression was induced by adenovirus AxCANCre and which were then further passaged 3 times.
5. 1d and 3d respectively indicate one day and three days after the  
15 induction of expression.
6. Vac1d and Vac3d respectively indicate cells one day and three days after the infection of vaccinia virus.
7. AraC1d and AraC3d respectively indicate cells one day and three days after the addition of AraC.
- 20 8. CHX 1d and CHX 3d respectively indicate cells one day and three days after the addition of protein synthesis inhibitor cycloheximide.

Figure 6 indicates photographs showing the result that was obtained by observing GFP expression after GFP-comprising F-deficient SeV cDNA (pSeV18<sup>+</sup>/ΔF-GFP) was transfected into LLC-MK2 cells in which  
25 F was not expressed (detection of RNP). In a control group, the F gene was shuffled with the NP gene at the 3' end, and then, SeV cDNA (F-shuffled SeV), in which GFP had been introduced into the F-deficient site, was used. The mark "all" indicates cells transfected with plasmids directing the expression of the NP gene,  
30 P gene, and L gene (pGEM/NP, pGEM/P, and pGEM/L) together with SeV cDNA at the same time; "cDNA" indicates cells transfected with cDNA (pSeV18<sup>+</sup>/ΔF-GFP) alone. For RNP transfection, P0 cells expressing GFP were collected; the cells (10<sup>7</sup> cells/ml) were suspended in OptiMEM (GIBCO BRL); 100 μl of lysate prepared after treating three times  
35 with freeze-thaw cycles was mixed with 25 μl of cationic liposome DOSPER (Boehringer Mannheim) and allowed to stand still at room

temperature for 15 minutes; and the mixture was added to cells (+ad) in which the expression of F had been induced to achieve the RNP transfection. Cells expressing Cre DNA recombinase, in which no recombinant adenovirus had been introduced, were used as a control group of cells (-ad). The result showed that GFP was expressed depending on the RNP formation of SeV in P0 in LLC-MK2 cells; and the F-deficient virus was amplified depending on the induction of expression of F in P1.

Figure 7 indicates photographs showing the result that was obtained by studying whether functional RNP reconstituted with F-deficient genomic cDNA could be rescued by the F-expressing helper cells and form the infective virion of the deficient virus. RNP/o represents cells overlaid with RNP; RNP/t represents cells that was transfected with RNP.

Figure 8 indicates photographs showing the evidence for the F-expressing cell-specific growth of the F-deficient virus. The lysate comprising functional RNP constructed from the genome lacking the gene was lipofected to the F-expressing cells as described in Example 2; and the culture supernatant was then recovered. This culture supernatant was added to the medium of the F-expressing cells to achieve the infection; on the third day, the culture supernatant was recovered and concurrently added to both F-expressing cells and cells that had not expressed F; and then the cells were cultured in the presence or absence of trypsin for three days. The result is shown here. The viruses were amplified only in the presence of trypsin in the F-expressing cells.

Figure 9 indicates photographs showing evidence for specific release of the F-deficient viruses to the culture supernatant after the introduction into F-expressing cells. The lysate comprising functional RNP constructed from the genome lacking the gene was lipofected to the F-expressing cells as described in Example 2 and then the culture supernatant was recovered. This culture supernatant was added to the medium of the F-expressing cells to achieve the infection; on the third day, the culture supernatant was recovered and concurrently added to both F-expressing cells and cells that did not express F; and then the cells were cultured in the presence or



absence of trypsin for three days. The bottom panel shows the result with supernatant of the cells that did not express F.

Figure 10 indicates photographs showing the result obtained by recovering viruses from the culture supernatant of the F-expressing cells, extracting the total RNA and performing Northern blot analysis using F and HN as probes to verify the genomic structure of virion recovered from the F-deficient cDNA. In the viruses recovered from the F-expressing cells, the HN gene was detected but the F gene was not detectable; and thus it was clarified that the F gene was not present in the viral genome.

Figure 11 indicates photographs showing the result of RT-PCR, which demonstrates that the GFP gene is present in the locus where F had been deleted, as in the construct of the cDNA. 1: +18-NP, for the confirmation of the presence of +18 NotI site. 2: M-GFP, for the confirmation of the presence of the GFP gene in the F gene-deficient region. 3: F gene, for the confirmation of the presence of the F gene. The genomic structures of wild type SeV and F-deficient GFP-expressing SeV are shown in the top panel. It was verified that the GFP gene was present in the F-deficient locus, +18-derived NotI site was present at the 3' end of NP and the F gene was absent in any part of the RNA genome.

Figure 12 indicates photographs that were obtained by the immuno-electron microscopic examination with gold colloid-bound IgG (anti-F, anti-HN) specifically reacting to F or HN of the virus. It was clarified that the spike-like structure of the virus envelope comprised F and HN proteins.

Figure 13 indicates diagrams showing the result of RT-PCR, which demonstrates that the structures of genes except the GFP gene were the same as those from the wild type.

Figure 14 indicates photographs showing the result obtained by examining the F-deficient virus particle morphology by electron microscopy. Like the wild-type virus particles, the F-deficient virus particles had helical RNP structure and spike-like structure inside.

Figure 15 indicates photographs showing the result of *in vitro* gene transfer to a variety of cells using an F-deficient SeV vector

with a high efficiency.

Figure 16 indicates diagrams showing the analytical result obtained after the introduction of the F-deficient SeV vector into primary bone marrow cells from mouse (BM c-kit+/-). Open bars represent PE-positive/GFP-negative; closed bars represent PE-positive/GFP-positive.

Figure 17 indicates photographs showing the result of *in vivo* administration of the vector into the rat cerebral ventricle.

Figure 18 indicates photographs showing the result obtained by using the culture supernatant comprising F-deficient SeV viruses recovered from the F-expressing cells to infect LLC-MK2 cells that do not express F, culturing the cells in the presence or absence of trypsin for three days to confirm the presence of viruses in the supernatant by HA assay.

Figure 19 is a photograph showing the result obtained by conducting HA assay of chorioallantoic fluids after a 2-day incubation of embryonated chicken egg that had been inoculated with chorioallantoic fluid (lanes 11 and 12) from HA-positive embryonated eggs in Figure 18B.

Figure 20 indicates photographs showing the result obtained by examining the virus liquid, which is HA-positive and has no infectivity, by immuno-electron microscopy. The presence of the virus particles was verified and it was found that the virion envelope was reactive to antibody recognizing HN protein labeled with gold colloid, but not reactive to antibody recognizing F protein labeled with gold colloid.

Figure 21 indicates photographs showing the result of transfection of F-deficient virus particles into cells.

Figure 22 indicates photographs showing the result of creation of cells co-expressing F and HN, which were evaluated by Western blotting. LLC/VacT7/pGEM/FHN represents cells obtained by transfecting vaccinia-infected LLC-MK2 cells with pGEM/FHN plasmid; LLC/VacT7 represents vaccinia-infected LLC-MK2 cells. LLCMK2/FHNmix represents LLC-MK2 cells in which the F and HN genes were introduced but not cloned. LLC/FHN represents LLC-MK2 cells in which the F and HN genes were introduced and the expression was induced

by adenovirus AxCAVCre (after 3 days); 1-13, 2-6, 2-16, 3-3, 3-18, 3-22, 4-3 and 5-9 are cell-line numbers (names) in the cloning.

Figure 23 indicates photographs showing the result for the confirmation of virus generation depending on the presence or absence  
5 pGEM/FHN. FHN-deficient GFP-expressing SeV cDNA, pGEM/NP, pGEM/P, pGEM/L, and pGEM/FHN were mixed and introduced into LLC-MK2 cells. 3 hours after the gene transfer, the medium was changed with MEM containing AraC and trypsin and then the cells were further cultured for three days. 2 days after the gene transfer, observation was  
10 carried out with a stereoscopic fluorescence microscope to evaluate the difference depending on the presence or absence of pGEM/FHN, and the virus generation was verified based on the spread of GFP-expressing cells. The result is shown here. When pGEM/FHN was added at the time of reconstitution, the spread of GFP-expressing  
15 cells was recognized; but when no pGEM/FHN was added, the GFP expression was observable merely in a single cell.

Figure 24 indicates photographs showing the result of reconstitution by RNP transfection and growth of FHN-deficient viruses. On the third day after the induction of expression, cells  
20 co-expressing FHN (12 wells) were lipofected by using P0 RNP overlay or DOSPER, and then GFP was observed after 4 days. When RNP transfection was conducted, the harvest of viruses was successful for P1 FHN-expressing cells as was for the F-deficient ones (top). The growth of the FHN-deficient viruses was verified after inoculating  
25 a liquid comprising the viruses to cells in which the expression of FHN protein was induced 6 hours or more after the infection with AxCANCre (bottom panel).

Figure 25 indicates photographs showing the result obtained after inoculating the liquid comprising viruses reconstituted from  
30 FHN-deficient GFP-expressing cDNA to LLC-MK2, LLC-MK2/F, LLC-MK2/HN, and LLC-MK2/FHN and culturing them in the presence or absence of the trypsin. The spread of cells expressing GFP protein was verified 3 days after the culture. The result is shown here. The expansion of GFP was observed only with LLC-MK2/FHN, and thus it was verified that  
35 the virus contained in the liquid was grown in a manner specific to FHN co-expression and dependent on trypsin.

Figure 26 is a photograph showing the result where the confirmation was carried out for the genomic structure of RNA derived from supernatant of the FHN-expressing cells.

5 Figure 27 is a photograph showing the result where the confirmation was carried out for the genomic structure of RNA derived from supernatant of the F-expressing cells infected with the FHN-deficient viruses.

10 Figure 28 is a diagram showing inactivation of vaccinia virus and T7 activity when psoralen concentration was varied in psoralen/UV irradiation.

Figure 29 is a diagram showing inactivation of vaccinia virus and T7 RNA polymerase activity when the duration of UV irradiation was varied in psoralen/UV irradiation.

15 Figure 30 indicates photographs showing a cytotoxicity (CPE) of vaccinia virus after psoralen/UV irradiation.  $3 \times 10^5$  LLC-MK2 cells were plated on a 6-well plate. After culturing overnight, the cells were infected with vaccinia virus at  $\text{moi}=2$ . After 24 hours, CPE was determined. The result of CPE with mock-treatment of vaccinia virus is shown in A; CPE after the treatment with vaccinia virus for 15, 20, or 30 minutes are shown in B, C, and D, respectively.

Figure 31 is a diagram indicating the influence of duration of UV treatment of vaccinia virus on the reconstitution efficiency of Sendai virus.

25 Figure 32 is a diagram indicating the titer of vaccinia virus capable of replicating that remained in the cells after the reconstitution experiment of Sendai virus.

Figure 33 is a photograph showing a result of Western blot analysis using anti-VSV-G antibody.

30 Figure 34 indicates a diagram showing results of flow cytometry analysis using anti-VSV-G antibody. It shows the result of analysis of LLC-MK2 cell line (L1) for the induction of VSV-G expression on the fourth day after AxCANCre infection ( $\text{moi}=0, 2.5, 5$ ). Primary antibody used was anti-VSV-G antibody (MoAb I-1); secondary antibody was FITC-labeled anti-mouse Ig.

35 Figure 35 indicates photographs showing a result where supernatants were recovered after the infection with altered amounts

of AxCANCre (MOI=0, 1.25, 2.5, 5, 10) and a constant amount of pseudo-type Sendai virus having a F gene-deficient genome, and further the supernatants were used to infect cells before VSV-G induction (-) and after induction (+), and cells expressing GFP were observed after 5 days.

Figure 36 indicates photographs showing the result obtained for the time course of virus production amount.

Figure 37 indicates photographs showing the result obtained by examining whether the infectivity is influenced by the treatment of pseudo-type Sendai virus having the F gene-deficient genome, which was established with the VSV-G-expressing cell line, and FHN-deficient Sendai virus treated with anti-VSV antibody.

Figure 38 indicates photographs showing the result where the expression of the GFP gene was tested as an index to determine the presence of production of the pseudo-type virus having VSV-G in its capsid after the infection of VSV-G gene-expressing cells LLCG-L1 with F and HN-deficient Sendai virus comprising the GFP gene.

Figure 39 indicates photographs showing the result confirming that viruses grown in the VSV-G gene-expressing cells were deficient in F and HN genes by Western analysis of protein in the extract of infected cells.

Figure 40 indicates photographs showing the result for the observation of GFP-expressing cells under a fluorescence microscope.

Figure 41 is a diagram showing the improvement in efficiency for the reconstitution of SeV/ $\Delta$ F-GFP by the combined use of the envelope-expressing plasmid and cell overlay. Considerable improvement was recognized at d3 to d4 (day 3 to day 4) of P0 (prior to passaging).

Figure 42 is a diagram showing the result where treatment conditions were evaluated for the reconstitution of SeV/ $\Delta$ F-GFP by the combined use of the envelope-expressing plasmid and cell overlay. GFP-positive cells represent the amount of virus reconstituted.

Figure 43 is a diagram showing the result where the rescue of F-deficient Sendai viruses from cDNA was tested. It shows the improvement in efficiency for the reconstitution of SeV/ $\Delta$ F-GFP by the combined use of the envelope-expressing plasmid and cell overlay:

All the tests were positive on the seventh day. However, the efficiency was evaluated on the third day where the probability of success was midrange.

Figure 44 indicates photographs showing the result of lacZ expression by LacZ-comprising F-deficient Sendai virus vector comprising no GFP.

Figure 45 indicates diagrams showing subcloning of Sendai virus genomic cDNA fragment (A) and structures of 5 Sendai virus genomic cDNAs constructed with newly introduced NotI site (B).

Figure 46 is a diagram showing structures of plasmids to be used for cloning to add NotI site, transcription initiation signal, intervening sequence, and transcription termination signal into SEAP.

Figure 47 indicates photographs showing the result of plaque assay of each Sendai virus vector. It shows partial fluorescence image in the plaque assay obtained by LAS1000.

Figure 48 is a diagram showing the result where altered expression levels of reporter gene (SEAP) were compared with one another among the respective Sendai virus vectors. The data of SeV18+/SEAP was taken as 100 and the respective values were indicated relative to it. It was found that the activity, namely the expression level, was decreased as the SEAP gene was placed more downstream.

Figure 49 indicates microscopic photographs showing the expression of GFP in P1 cells co-expressing FHN.

Figure 50 indicates photographs showing the result of Western blot analysis of the extracts from cells infected with VSV-G pseudo-type SeV/ $\Delta$ F:GFP using anti-F antibody (anti-F), anti-HN antibody (anti-HN), and anti-Sendai virus antibody (anti-SeV).

Figure 51 indicates photographs showing GFP fluorescence from F- and HN-deficient cells infected with VSV-G pseudo-type SeV in the presence or absence of a neutralizing antibody (VGV antibody).

Figure 52 indicates photographs showing results of Western analysis for VSV-G pseudo-type Sendai viruses having F gene-deficient or F gene- and HN gene-deficient genome, which were fractionated by density gradient ultracentrifugation.

Figure 53 indicates photographs showing hematoadsorption test mediated with Sendai viruses having F gene-deficient genome, or VSV-G

pseudo-type Sendai viruses having F gene-deficient or F gene- and HN gene-deficient genome.

Figure 54 indicates diagrams showing the specificity of infection to culture cells of Sendai virus having F gene-deficient genome or VSV-G pseudo-type Sendai virus.

Figure 55 indicates photographs showing the confirmation of the structures of NGF-expressing F-deficient Sendai virus (NGF/SeV/ $\Delta$ F).

Figure 56 is a diagram showing the activity of NGF expressed by the NGF-comprising cells infected with F-deficient SeV. With the initiation of culture, diluted supernatant of SeV-infected cells or NGF protein (control) was added to a dissociated culture of primary chicken dorsal root ganglion (DRG) neurons. After three days, the viable cells were counted by using mitochondrial reduction activity as an index (n=3). The quantity of culture supernatant added corresponded to 1000-fold dilution.

Figure 57 indicates photographs showing the activity of NGF expressed by the NGF-comprising cells infected with F-deficient SeV. With the initiation of culture, diluted supernatant of SeV-infected cells or NGF protein (control) was added to a dissociated culture of primary chicken dorsal root ganglion (DRG) neurons. After three days, the samples were observed under a microscope,

- A) control (without NGF);
- B) addition of NGF protein (10 ng/mL);
- C) addition of culture supernatant (100-fold diluted) of NGF/SeV infected cells;
- D) addition of culture supernatant (100-fold diluted) of NGF/SeV infected cells;
- E) addition of culture supernatant (100-fold diluted) of NGF/SeV/ $\Delta$ F infected cells, and;
- F) addition of culture supernatant (100-fold diluted) of NGF/SeV/ $\Delta$ F-GFP infected cells.

Figure 58 is a photograph showing moi of AxCANCre and the expression level of F protein.

Figure 59 indicates photographs showing the expression of LLC-MK2/F by AxCANCre.

Figure 60 is a photograph showing the durability of expression

over the passages.

Figure 61 indicates photographs showing the localization of F protein over the passages.

5 Figure 62 is a diagram showing the correlation between GFP-CIU and anti-SeV-CIU.

Figure 63 indicates a diagram showing structures of genes encoding F-deficient SeV and additional type SeV genomes having GFP and/or SEAP genes.

10 Figure 64 indicates photographs showing micrographs showing the expression of GFP after cells continuously expressing F protein (LLC-MK2/F7/A) were infected with SeV18+/ $\Delta$ F-GFP and cultured for 6 days at 32°C or 37°C.

15 Figure 65 indicates a photograph showing the result that was obtained by culturing, at 32°C or 37°C in serum-free MEM containing trypsin, cells continuously expressing SeV-F protein (LLC-MK2/F7/A) and by semi-quantitatively measuring the expression level of F protein by Western-blotting over time.

20 Figure 66 indicates photographs showing micrographs showing the expression of GFP after LLC-MK2 cells were infected with SeV18+GFP or SeV18+/ $\Delta$ F-GFP at m.o.i.=3 and cultured for 3 days at 32°C, 37°C, or 38°C.

25 Figure 67 indicates diagrams showing hemagglutination activity (HA activity) of the culture supernatants that were sampled over time (the media were exchanged for new ones at the same time) after LLC-MK2 cells were infected with SeV18+GFP or SeV18+/ $\Delta$ F-GFP at m.o.i.=3 and cultured at 32°C, 37°C, or 38°C.

30 Figure 68 indicates photographs showing ratios of M protein in cells to that in virus-like particles. The ratios were measured by Western-blotting with anti-M protein antibody by recovering the culture supernatants and the cells that were obtained after LLC-MK2 cells were infected with SeV18+GFP or SeV18+/ $\Delta$ F-GFP at m.o.i.= 3 and cultured for 2 days at 37°C and by using 1/10 equivalents of 1 well of 6-well-plate culture per lane.

35 Figure 69 indicates a diagram showing the construction scheme for M-deficient SeV genome cDNA having an EGFP gene.

Figure 70 indicates a diagram showing the construction scheme



for both F- and M-deficient SeV genome cDNA.

Figure 71 indicates a diagram showing structures of the constructed F- and/or M-deficient SeV genes.

Figure 72 indicates a diagram showing the construction scheme  
5 for the M gene-expressing plasmid having a hygromycin resistance gene.

Figure 73 indicates photographs showing the result that was obtained by semi-quantitatively comparing the expression of M and F proteins by Western-blotting after cells inducibly expressing the cloned M (and F) proteins were infected with Cre DNA  
10 recombinase-expressing recombinant adenovirus (AxCANCre).

Figure 74 indicates photographs showing viral reconstitution of M-deficient SeV (SeV18+/ $\Delta$ M-GFP) using helper cell (LLC-MK2/F7/M) clones #18 and #62.

Figure 75 indicates a diagram showing the virus productivity  
15 of SeV18+/ $\Delta$ M-GFP (time courses of CIU and HAU).

Figure 76 indicates photographs and a diagram showing the result of RT-PCR for confirming the gene structure in virions of SeV18+/ $\Delta$ M-GFP.

Figure 77 indicates photographs showing the result of  
20 comparison between SeV18+GFP and SeV18+/ $\Delta$ F-GFP that was obtained, to confirm the virus structure of SeV18+/ $\Delta$ M-GFP, by carrying out Western-blotting for virus proteins in infected LLC-MK2 cells and the culture supernatants.

Figure 78 indicates a photograph showing the quantitative  
25 comparison (by preparing dilution series and by carrying out Western-blotting) of virus-derived proteins in the culture supernatants of LLC-MK2 cells infected with SeV18+/ $\Delta$ M-GFP and SeV18+/ $\Delta$ F-GFP.

Figure 79 indicates a diagram showing HA activity of the culture  
30 supernatants that were recovered over time after LLC-MK2 cells were infected with SeV18+/ $\Delta$ M-GFP or SeV18+/ $\Delta$ F-GFP at m.o.i.=3.

Figure 80 indicates photographs showing fluorescence  
micrographs 4 days after LLC-MK2 cells were infected with SeV18+/ $\Delta$ M-GFP or SeV18+/ $\Delta$ F-GFP at m.o.i.=3.

Figure 81 indicates photographs showing fluorescence  
35 micrographs 2 days after LLC-MK2 cells were infected using cationic

liposomes (Dosper) with the culture supernatants that were recovered 5 days after LLC-MK2 cells were infected with SeV18+/ $\Delta$ M-GFP or SeV18+/ $\Delta$ F-GFP at m.o.i.=3.

Figure 82 indicates photographs showing viral reconstitution of F- and M-deficient SeV (SeV18+/ $\Delta$ M $\Delta$ F-GFP).

Figure 83 indicates photographs showing fluorescence micrographs 3 days and 5 days after both M- and F-expressing cells (LLC-MK2/F7/M62/A) were infected with SeV18+/ $\Delta$ M-GFP or SeV18+/ $\Delta$ F-GFP.

Figure 84 represents a construction scheme for the vector that induces the M or F gene expression and has the Zeocin selection marker.

Figure 85 shows the expression of M and F proteins in M and F expressing helper cells.

Figure 86 represents photographs showing the GFP expression in cells transfected with M and F-deficient SeV having GFP gene.

Figure 87 is a graph showing the virus production from cells producing M and F-deficient SeV having GFP gene.

Figure 88 represents the genome structure of M and F-deficient SeV confirmed by RT-PCR. "dF" represents SeV18+/ $\Delta$ F-GFP, "dM" SeV18+/ $\Delta$ M-GFP, and "dM $\Delta$ F" SeV18+/ $\Delta$ M $\Delta$ F-GFP, respectively.

Figure 89 represents the results of Western blot analyses confirming deficiency of the expression of M and F proteins in cells transfected with M and F-deficient SeV.

Figure 90 is a graph showing the results of HA assay for examining the presence or absence of the secondarily released virus particles from cells transfected with M and F-deficient SeV.

Figure 91 represents photographs showing the results of examining the presence or absence of the secondarily released virus particles from cells transfected with M and F-deficient SeV. The examination was performed by transfecting cells with the culture supernatant of the M and F-deficient SeV-transfected cells.

Figure 92 represents photographs showing the infectivity of M and F-deficient SeV and M-deficient SeV against cerebral cortex nerve cells.

Figure 93 represents photographs showing the expression of the transferred gene after the *in vivo* administration of M and F-deficient

SeV and M-deficient SeV into the gerbil brain.

Figure 94 is a series of graphs showing the moi-dependent cytotoxicity of M and F-deficient SeV and M-deficient SeV. "Cont." represents SeV having the replication ability (SeV18+GFP), "dF" SeV18+/ $\Delta$ F-GFP, "dM" SeV18+/ $\Delta$ M-GFP, and "dMdF" SeV18+/ $\Delta$ M $\Delta$ F-GFP.

#### Best Mode for Carrying out the Invention

The present invention is illustrated in detail below with reference to Examples, but is not to be construed as being limited thereto. All the references cited herein are incorporated by reference.

##### [Example 1] Construction of F-deficient Sendai virus

##### <1> Construction of F-deficient SeV genomic cDNA and F-expressing plasmid

The full-length genomic cDNA of Sendai virus (SeV), pSeV18<sup>+</sup> b(+) (Hasan, M. K. et al., 1997, J. General Virology 78: 2813-2820) ("pSeV18<sup>+</sup> b(+)") is also referred to as "pSeV18<sup>+</sup>") was digested with SphI/KpnI, and the resulting fragment (14673 bp) was recovered and cloned into plasmid pUC18 to generate pUC18/KS. The F-disrupted site was constructed on this pUC18/KS. The F gene disruption was performed by the combined use of PCR-ligation method, and as a result, the ORF for the F gene (ATG-TGA=1698 bp) was removed; thus atgcatgccggcagatga (SEQ ID NO: 1) was ligated to it to construct the F-deficient SeV genomic cDNA (pSeV18<sup>+</sup>/ $\Delta$ F). In PCR, a PCR product generated by using a primer pair (forward: 5'-gttgagtactgcaagagc/SEQ ID NO: 2, reverse: 5'-tttgccggcatgcatgtttcccaaggggagagttttgcaacc/SEQ ID NO: 3) was ligated upstream of F and another PCR product generated by using a primer pair (forward: 5'-atgcatgccggcagatga/SEQ ID NO: 4, reverse: 5'-tgggtgaatgagagaatcagc/SEQ ID NO: 5) was ligated downstream of the F gene at EcoT22I site. The resulting plasmid was digested with SacI and SalI, and then the fragment (4931 bp) spanning the region comprising the site where F is disrupted was recovered and cloned into pUC18 to generate pUC18/dFSS. This pUC18/dFSS was digested with DraIII. The resulting fragment was recovered and substituted with a DraIII fragment from the region comprising the F gene of pSeV18<sup>+</sup>; and the ligation was carried out to generate plasmid pSeV18<sup>+</sup>/ $\Delta$ F.

Further, in order to construct a cDNA (pSeV18<sup>+</sup>/ΔF-GFP) in which the EGFP gene has been introduced at the site where F was disrupted, the EGFP gene was amplified by PCR. To set the EGFP gene with a multiple of 6 (Hausmann, S. et al., RNA 2, 1033-1045 (1996)), PCR was carried out with an NsiI-tailed primer (5'-atgcatatggtgatgcggttttggcagtac: SEQ ID NO: 6) for the 5' end and an NgoMIV-tailed primer (5'-Tgccggctattattacttgtacagctcgtc: SEQ ID NO: 7) for the 3' end. The PCR products were digested with restriction enzymes NsiI and NgoMIV, and then the fragment was recovered from the gel; the fragment was ligated at the site of pUC18/dFSS between NsiI and NgoMIV restriction enzyme sites where the disrupted F is located and the sequence was determined. A DraIII fragment comprising the EGFP gene was removed and recovered from the site, and substituted for a DraIII fragment in the region comprising the F gene of pSeV18<sup>+</sup>; then ligation was carried out to obtain plasmid pSeV18<sup>+</sup>/ΔF-GFP.

On the other hand, Cre-loxP-inducible expression plasmid for F gene expression was constructed by amplifying the SeV F gene by PCR, confirming the sequence, and inserting into the unique site SwaI of plasmid pCALNdLw (Arai et al., J. Virology 72, 1998, p1115-1121), in which the expression of gene products has been designed to be induced by Cre DNA recombinase, to obtain plasmid pCALNdLw/F.

<2> Preparation of helper cells inducing the expression of SeV-F protein

To recover infectious virus particles from F-deficient genome, a helper cell strain expressing SeV-F protein was established. The cell utilized was LLC-MK2 cell that is commonly used for the growth of SeV and is a cell strain derived from monkey kidney. The LLC-MK2 cells were cultured in MEM containing 10% heat-treated inactivated fetal bovine serum (FBS), sodium penicillin G (50 units/ml), and streptomycin (50 μg/ml) at 37°C under 5% CO<sub>2</sub> gas. Because SeV-F gene product is cytotoxic, the above-mentioned plasmid pCALNdLw/F designed to induce the expression of F gene product through Cre DNA recombinase was introduced into LLC-MK2 cells by calcium phosphate method (mammalian transfection kit (Stratagene)) according to the gene transfer protocol.

10 µg of plasmid pCALNdLw/F was introduced into LLC-MK2 cells grown to be 40% confluent in a 10-cm plate, and the cells were cultured in 10 ml of MEM containing 10% FBS at 37°C under 5% CO<sub>2</sub> for 24 hours in an incubator. After 24 hours, the cells were scraped off, and  
5 suspended in 10 ml medium; then the cells were plated on 5 dishes with 10-cm diameter (one plate with 5 ml; 2 plates with 2 ml; 2 plates with 0.2 ml) in MEM containing 10 ml of 10% FBS and 1200 µg/ml G418 (GIBCO-BRL) for the cultivation. The culture was continued for 14  
10 days while the medium was changed at 2-day intervals, to select cell lines in which the gene has been introduced stably. 30 cell strains were recovered as G418-resistant cells grown in the medium by using cloning rings. Each clone was cultured to be confluent in 10-cm plates.

After the infection of each clone with recombinant adenovirus  
15 AxCANCre expressing Cre DNA recombinase, the cells were tested for the expression of SeV-F protein by Western blotting using anti-SeV-F protein monoclonal IgG (f236; J. Biochem. 123: 1064-1072) as follows.

After grown to be confluent in a 6-cm dish, each clone was infected with adenovirus AxCANCre at moi=3 according to the method  
20 of Saito et al., (Saito et al., Nucl. Acids Res. 23: 3816-3821 (1995); Arai, T. et al., J Virol 72, 1115-1121 (1998)). After the infection, the cells were cultured for 3 days. The culture supernatant was discarded and the cells were washed twice with PBS buffer, scraped  
off with a scraper and were collected by centrifugation at 1500x g  
25 for five minutes.

The cells are kept at -80°C and can be thawed when used. The cells collected were suspended in 150 µl PBS buffer, and equal amount of 2x Tris-SDS-BME sample loading buffer (0.625 M Tris, pH 6.8, 5% SDS, 25% 2-ME, 50% glycerol, 0.025% BPB; Owl) was added thereto. The  
30 mixture was heat-treated at 98°C for 3 minutes and then used as a sample for electrophoresis. The sample (1x 10<sup>5</sup> cells/lane) was fractionated by electrophoresis in an SDS-polyacrylamide gel (Multi Gel 10/20, Daiichi Pure Chemicals). The fractionated proteins were transferred onto a PVDF transfer membrane (Immobilon-P transfer  
35 membranes; Millipore) by semi-dry blotting. The transfer was carried out under a constant current of 1 mA/cm<sup>2</sup> for 1 hour onto the transfer

membrane that had been soaked in 100% methanol for 30 seconds and then in water for 30 minutes.

The transfer membrane was shaken in a blocking solution containing 0.05% Tween20 and 1% BSA (BlockAce; Snow Brand Milk Products) for one hour, and then it was incubated at room temperature for 2 hours with an anti-SeV-F antibody (f236) which had been diluted 1000-folds with a blocking solution containing 0.05% Tween 20 and 1% BSA. The transfer membrane was washed 3 times in 20 ml of PBS-0.1% Tween20 while being shaken for 5 minutes and then it was washed in PBS buffer while being shaken for 5 minutes. The transfer membrane was incubated at room temperature for one hour in 10 ml of peroxidase-conjugated anti-mouse IgG antibody (Goat anti-mouse IgG; Zymed) diluted 2000-fold with the blocking solution containing 0.05% Tween 20 and 1% BSA. The transfer membrane was washed 3 times with 20 ml of PBS-0.1% Tween20 while being shaken for 5 minutes, and then it was washed in PBS buffer while being shaken for 5 minutes.

Detections were carried out for proteins cross-reacting to the anti-SeV-F antibody on the transfer membrane by chemiluminescence method (ECL western blotting detection reagents; Amersham). The result is shown in Figure 1. The SeV-F expression specific to AxCANCre infection was detected to confirm the generation of LLC-MK2 cells that induce expression of a SeV-F gene product.

One of the several resulting cell lines, LLC-MK2/F7 cell, was analyzed by flow cytometry with an anti-SeV-F antibody (Figure 2). Specifically,  $1 \times 10^5$  cells were precipitated by centrifugation at 15,000 rpm at 4°C for 5 minutes, washed with 200  $\mu$ l PBS, and allowed to react in PBS for FACS (NIKKEN CHEMICALS) containing 100-fold diluted anti-F monoclonal antibody (f236), 0.05% sodium azide, 2% FCS at 4°C for 1 hour in a dark place. The cells were again precipitated at 15,000 rpm at 4°C for 5 minutes, washed with 200  $\mu$ l PBS, and then allowed to react to FITC-labeled anti-mouse IgG (CAPPEL) of 1  $\mu$ g/ml on ice for 30 minutes. Then the cells were again washed with 200  $\mu$ l PBS, and then precipitated by centrifugation at 15,000 rpm at 4°C for 5 minutes. The cells were suspended in 1 ml of PBS for FACS and then analyzed by using EPICS ELITE (Coulter) argon laser at an excitation wavelength of 488 nm and at a fluorescence wavelength

of 525 nm. The result showed that LLC-MK2/F7 exhibited a high reactivity to the antibody in a manner specific to the induction of SeV-F gene expression, and thus it was verified that SeV-F protein was expressed on the cell surface.

5

[Example 2] Confirmation of function of SeV-F protein expressed by helper cells

It was tested whether or not SeV-F protein, of which expression was induced by helper cells, retained the original protein function.

10

After plating on a 6-cm dish and grown to be confluent, LLC-MK2/F7 cells were infected with adenovirus AxCANCre at moi=3 according to the method of Saito et al. (described above). Then, the cells were cultured in MEM (serum free) containing trypsin (7.5 µg/ml; GIBCO-BRL) at 37°C under 5% CO<sub>2</sub> in an incubator for three days.

15

The culture supernatant was discarded and the cells were washed twice with PBS buffer, scraped off with a scraper, and collected by centrifugation at 1500 x g for five minutes. The cleavage of expressed F protein by trypsin was verified by Western blotting as described above (Figure 3). SeV-F protein is synthesized as F0 that is a non-active protein precursor, and then the precursor is activated after being digested into two subunits F1 and F2 by proteolysis with trypsin. LLC-MK2/F7 cells after the induction of F protein expression thus, like ordinary cells, continues to express F protein, even after being passaged, and no cytotoxicity mediated by the

20

25

expressed F protein was observed as well as no cell fusion of F protein-expressing cells was observed. However, when SeV-HN expression plasmid (pCAG/SeV-HN) was transfected into the F-expressing cells and the cells were cultured in MEM containing trypsin for 3 days, cell fusion was frequently observed. The expression of HN on the cell surface was confirmed in an experiment using erythrocyte adsorption onto the cell surface (Hematoadsorption assay; Had assay) (Figure 4). Specifically, 1% chicken erythrocytes were added to the culture cells at a concentration of 1 ml/dish and the mixture was allowed to stand still at 4°C for 10 minutes. The

30

35

cells were washed 3 times with PBS buffer, and then colonies of erythrocytes on the cell surface were observed. Cell fusion was

recognized for cells on which erythrocytes aggregated; cell fusion was found to be induced through the interaction of F protein with HN; and thus it was demonstrated that F protein, the expression of which was sustained in LLC-MK2/F7, retained the original function thereof.

[Example 3] Functional RNP having F-deficient genome and formation of virions

To recover virions from the deficient viruses, it is necessary to use cells expressing the deficient protein. Thus, the recovery of the deficient viruses was attempted with cells expressing the deficient protein, but it was revealed that the expression of F protein by the helper cell line stopped rapidly due to the vaccinia viruses used in the reconstitution of F-deficient SeV (Figure 5) and thus the virus reconstitution based on the direct supply of F protein from the helper cell line failed. It has been reported that replication capability of vaccinia virus is inactivated, but the activity of T7 expression is not impaired by the treatment of vaccinia virus with ultraviolet light of long wavelengths (long-wave UV) in the presence of added psoralen (PLWUV treatment) (Tsunget al., J Virol 70, 165-171, 1996). Thus, virus reconstitution was attempted by using PLWUV-treated vaccinia virus (PLWUV-VacT7). UV Stratalinker 2400 (Catalog NO. 400676 (100V); Stratagene, La Jolla, CA, USA) equipped with five 15-Watt bulbs was used for ultraviolet light irradiation. The result showed that the expression of F protein was inhibited from the F-expressing cells used in the reconstitution, but vaccinia viruses were hardly grown in the presence of AraC after lysate from the cells reconstituted with this PLWUV-VacT7 was infected to the helper cells, and it was also found that the expression of F protein by the helper cell line was hardly influenced. Further, this reconstitution of wild type SeV using this PLWUV-VacT7 enables the recovery of viruses from even  $10^3$  cells, whereas by previous methods, this was not possible unless  $10^5$  or more cells were there, and thus the efficiency of virus reconstitution was greatly improved. Thus, reconstitution of F-deficient SeV virus was attempted by using this method.



<Reconstitution and amplification of F-deficient SeV virus>

The expression of GFP was observed after transfecting LLC-MK2 cells with the above-mentioned pSeV18<sup>+</sup>/ΔF-GFP in which the enhanced green fluorescent protein (EGFP) gene had been introduced as a reporter into the site where F had been disrupted according to the 6n rule in the manner as described below. It was also tested for the influence of the presence of virus-derived genes NP, P, and L that are three components required for the formation of RNP.

LLC-MK2 cells were plated on a 100-mm Petri-dish at a concentration of  $5 \times 10^6$  cells/dish and were cultured for 24 hours. After the culture was completed, the cells were treated with psoralen and ultraviolet light of long wavelengths (365 nm) for 20 minutes, and the cells were infected with recombinant vaccinia virus expressing T7 RNA polymerase (Fuerst, T.R. et al., Proc. Natl. Acad. Sci. USA 83, 8122-8126 (1986)) at room temperature for one hour (moi=2) (moi=2 to 3; preferably moi=2). After the cells were washed 3 times, plasmids pSeV18<sup>+</sup>/ΔF-GFP, pGEM/NP, pGEM/P, and pGEM/L (Kato, A. et al., Genes cells 1, 569-579 (1996)) were respectively suspended in quantities of 12 μg, 4 μg, 2 μg, and 4 μg /dish in OptiMEM (GIBCO); SuperFect transfection reagent (1 μg DNA/5 μl SuperFect; QIAGEN) was added thereto; the mixtures were allowed to stand still at room temperature for 10 minutes; then they are added to 3 ml of OptiMEM containing 3% FBS; cells were added thereto and cultured. The same experiment was carried out using wild-type SeV genomic cDNA (pSeV(+)) (Kato, A. et al., Genes cells 1, 569-579 (1996)) as a control instead of pSeV18<sup>+</sup>/ΔF-GFP. After culturing for 3 hours, the cells were washed twice with MEM containing no serum, and then cultured in MEM containing cytosine β-D-arabinofuranoside (AraC, 40 μg/ml; Sigma) and trypsin (7.5 μg/ml; GIBCO) for 70 hours. These cells were harvested, and the pellet was suspended in OptiMEM ( $10^7$  cells/ml). After freeze-and-thaw treatment was repeated 3 times, the cells were mixed with lipofection reagent DOSPER (Boehringer Mannheim) ( $10^6$  cells/25 μl DOSPER) and allowed to stand still at room temperature for 15 minutes. Then F-expressing LLC-MK2/F7 cell line ( $10^6$  cells /well in 12-well plate) was transfected, and the cells were cultured in MEM containing no serum (containing 40 μg/ml AraC and 7.5 μg/ml trypsin).

The result showed that the expression of GFP was recognized only when all the three components, NP, P, and L derived from the virus are present and the deficient virus RNP expressing foreign genes can be generated (Figure 6).

5 <Confirmation of F-deficient virions>

It was tested whether the functional RNP reconstituted by F-deficient genomic cDNA by the method as described above could be rescued by the F-expressing helper cells and form infective virions of F-deficient virus. Cell lysates were mixed with cationic liposome; the lysates were prepared by freeze/thaw from cells reconstituted under conditions in which functional RNP is formed (condition where pSeV18<sup>+</sup>/ΔF-GFP, pGEM/NP, pGEM/P, and pGEM/L are transfected at the same time) or conditions under which functional RNP is not formed (conditions in which two plasmids, pSeV18<sup>+</sup>/ΔF-GFP and pGEM/NP, are transfected) as described above; the lysates were lipofected into F-expressing cells and non-expressing cells; the generation of virus particles was observed based on the expansion of the distribution of GFP-expressing cells. The result showed that the expansion of distribution of GFP-expressing cells was recognized only when the introduction to the F-expressing cells was carried out by using a lysate obtained under condition in which functional RNP is reconstituted (Figure 7). Furthermore, even in plaque assay, the plaque formation was seen only under the same conditions. From these results, it was revealed that functional RNPs generated from F-deficient virus genome were further converted into infective virus particles in the presence of F protein derived from F-expressing cells and the particles were released from the cells.

The demonstration of the presence of infective F-deficient virions in the culture supernatant was carried out by the following experiment. The lysate comprising the functional RNP constructed from the F gene deficient genome was lipofected to F-expressing cells as described in Example 2, and the culture supernatant was recovered. This culture supernatant was added to the medium of F-expressing cells to achieve the infection; on the third day, the culture supernatant was recovered and concurrently added to both F-expressing cells and cells that did not express F; and then the cells were cultured in

the presence or absence of trypsin for three days. In F-expressing cells, viruses were amplified only in the presence of trypsin (Figure 8). It was also revealed that non-infectious virus particles were released into the supernatant of cells that do not express F (in the bottom panel of Figure 9) or from F-expressing cells cultured in the absence of trypsin. A summary of the descriptions above is as follows: the growth of F-deficient GFP-expressing viruses is specific to F-expressing cells and depends on the proteolysis with trypsin. The titer of infective F-deficient Sendai virus thus grown ranged from  $0.5 \times 10^7$  to  $1 \times 10^7$  CIU/ml.

#### [Example 4] Analysis of F-deficient GFP-expressing virus

In order to confirm the genomic structure of virions recovered from F-deficient cDNA, viruses were recovered from the culture supernatant of the F-expressing cells, the total RNA was extracted and then Northern blot analysis was conducted by using F and HN as probes. The result showed that the HN gene was detectable, but the F gene was not detectable in the viruses harvested from the F-expressing cells, and it was clarified that the F gene was not present in the viral genome (Figure 10). Further, by RT-PCR, it was confirmed that the GFP gene was present in the deleted locus for F as shown in the construction of the cDNA (Figure 11) and that the structures of other genes were the same as those from the wild type. Based on the findings above, it was shown that no rearrangement of the genome had occurred during the virus reconstitution. In addition, the morphology of recovered F-deficient virus particles was examined by electron microscopy. Like the wild type virus, F-deficient virus particles had the helical RNP structure and spike-like structure inside (Figure 14). Further, the viruses were examined by immuno-electron microscopy with gold colloid-conjugated IgG (anti-F, anti-HN) specifically reacting to F or HN. The result showed that the spike-like structure of the envelope of the virus comprised F and HN proteins (Figure 12), which demonstrated that F protein produced by the helper cells was efficiently incorporated into the virions. The result will be described below in detail.

<Extraction of total RNA, Northern blot analysis, and RT-PCR>

Total RNA was extracted from culture supernatant obtained 3 days after the infection of F-expressing cell LLC-MK2/F7 with the viruses by using QIAamp Viral RNA mini kit (QIAGEN) according to the protocol. The purified total RNA (5 µg) was separated by electrophoresis in a 1% denaturing agarose gel containing formaldehyde, and then transferred onto a Hybond-N+ membrane in a vacuum blotting device (Amersham-Pharmacia). The prepared membrane was fixed with 0.05 M NaOH, rinsed with 2-fold diluted SSC buffer (Nacalai tesque), and then was subjected to pre-hybridization in a hybridization solution (Boehringer Mannheim) for 30 minutes; a probe for the F or HN gene prepared by random prime DNA labeling (DIG DNA Labeling Kit; Boehringer Mannheim) using digoxigenin (DIG)-dUTP (alkaline sensitive) was added thereto and then hybridization was performed for 16 hours. Then, the membrane was washed, and allowed to react to alkaline phosphatase-conjugated anti-DIG antibody (anti-digoxigenin-AP); the analysis was carried out by using a DIG detection kit. The result showed that the HN gene was detectable but the F gene was not detectable in the viruses harvested from the F-expressing cells, and it was clarified that the F gene was not present in the viral genome (Figure 10).

Further, detailed analysis was carried out by RT-PCR. In the RT-PCR, first strand cDNA was synthesized from the purified virus RNA by using SUPERSRIPTII Preamplification System (GIBCO-BRL) according to the protocol; the following PCR condition was employed with LA PCR kit (TAKARA ver2.1): 94°C/3 min; 30 cycles for the amplification of 94°C/45 sec, 55°C/45 sec, 72°C/90 sec; incubation at 72°C for 10 minutes; then the sample was electrophoresed in a 2% agarose gel at 100 v for 30 minutes, the gel was stained with ethidium bromide for a photographic image. Primers used to confirm the M gene and EGFP inserted into the F-deficient site were forward 1: 5'-atcagagacctgcgacaatgc (SEQ ID NO: 8) and reverse 1: 5'-aagtcgtgctgcttcatgtgg (SEQ ID NO: 9); primers used to confirm EGFP inserted into the F-deficient site and the HN gene were forward 2: 5'-acaaccactacctgagcaccacgtc (SEQ ID NO: 10) and reverse 2: 5'-gcctaacacatccagagatcg (SEQ ID NO: 11); and the junction between the M gene and HN gene was confirmed by using forward 3:

5'-acattcatgagtcagctcgc (SEQ ID NO: 12) and reverse 2 primer (SEQ ID NO: 11). The result showed that the GFP gene was present in the deficient locus for F as shown in the construction of the cDNA (Figure 11) and that the structures of other genes were the same as those from the wild type (Figure 13). From the findings shown above, it is clarified that no rearrangement of the genome had resulted during the virus reconstitution.

<Electron microscopic analysis with gold colloid-conjugated immunoglobulin>

10       The morphology of recovered F-deficient virus particles were examined by electron microscopy. First, culture supernatant of cells infected with the deficient viruses was centrifuged at 28,000 rpm for 30 minutes to obtain a virus pellet; then the pellet was re-suspended in 10-fold diluted PBS at a concentration of  $1 \times 10^9$  HAU/ml; one drop of the suspension was dropped on a microgrid with a supporting filter and then the grid was dried at room temperature; the grid was treated with PBS containing 3.7% formalin for 15 minutes for fixation and then pre-treated with PBS solution containing 0.1% BSA for 30 minutes; further, anti-F monoclonal antibody (f236) or anti-HN monoclonal antibody (Miura, N. et al., Exp. Cell Res. (1982) 141: 409-420) diluted 200-folds with the same solution was dropped on the grid and allowed to react under a moist condition for 60 minutes. Subsequently, the grid was washed with PBS, and then gold colloid-conjugated anti-mouse IgG antibody diluted 200-folds was dropped and allowed to react under a moist condition for 60 minutes. Subsequently, the grid was washed with PBS and then with distilled sterile water, and air-dried at room temperature; 4% uranium acetate solution was placed on the grid for the staining for 2 minutes and the grid was dried; the sample was observed and photographed in a JEM-1200EXII electron microscope (JEOL.). The result showed that the spike-like structure of the envelope of the virus comprised F and HN proteins (Figure 12), which demonstrated that F protein produced by the helper cells was efficiently incorporated into the virions. In addition, like the wild type virus, F-deficient virus particles had a helical RNP structure and a spike-like structure inside (Figure 14).

[Example 5] High-efficiency gene transfer to a variety of cells via F-deficient SeV vector *in vitro*

<Introduction into primary culture cells of rat cerebral cortex nerve  
5 cells>

Primary culture cells of rat cerebral cortex neurons were prepared and cultured as follows: an SD rat (SPF/VAF Crj: CD, female, 332 g, up to 9-week old; Charles River) on the eighteenth day of pregnancy was deeply anesthetized by diethyl ether, and then  
10 euthanized by bloodletting from axillary arteries. The fetuses were removed from the uterus after abdominal section. The cranial skin and bones were cut and the brains were taken out. The cerebral hemispheres were transferred under a stereoscopic microscope to a working solution DMEM (containing 5% horse serum, 5% calf serum and  
15 10% DMSO); they were sliced and an ice-cold papain solution (1.5 U, 0.2 mg of cysteine, 0.2 mg of bovine serum albumin, 5 mg glucose, DNase of 0.1 mg/ml) was added thereto; the solution containing the sliced tissues was incubated for 15 minutes while shaking by inverting the vial every 5 minutes at 32°C. After it was verified that the  
20 suspension became turbid enough and the tissue sections became translucent, the tissue sections were crushed into small pieces by pipetting. The suspension was centrifuged at 1200 rpm at 32°C for 5 minutes, and then the cells were re-suspended in B27-supplemented neural basal medium (GIBCO-BRL, Burlington, Ontario, Canada). The  
25 cells were plated on a plate coated with poly-D-lysine (Becton Dickinson Labware, Bedford, MA, U.S.A.) at a density of  $1 \times 10^5$  cells/dish and then cultured at 37°C under 5% CO<sub>2</sub>.

After the primary culture of nerve cells from cerebral cortex ( $5 \times 10^5$ /well) were cultured for 5 days, the cells were infected with  
30 F-deficient SeV vector (moi=5) and further cultured for three days. The cells were fixed in a fixing solution containing 1% paraformaldehyde, 5% goat serum, and 0.5% Triton-X at room temperature for five minutes. Blocking reaction was carried out for the cells by using BlockAce (Snow Brand Milk Products) at room temperature for  
35 2 hours, and then incubated with 500-fold diluted goat anti-rat microtubule-associated protein 2 (MAP-2) (Boehringer) IgG at room

temperature for one hour. Further, the cells were washed three times with PBS(-) every 15 minutes and then were incubated with cys3-conjugated anti-mouse IgG diluted 100-folds with 5% goat serum/PBS at room temperature for one hour. Further, after the cells were washed three times with PBS(-) every 15 minutes, Vectashield mounting medium (Vector Laboratories, Burlingame, U.S.A.) was added to the cells; the cells, which had been double-stained with MAP-2 immuno staining and GFP fluorescence, were fluorescently observed by using a confocal microscope (Nippon Bio-Rad MRC 1024, Japan) and an inverted microscope Nikon Diaphot 300 equipped with excitation band-pass filter of 470-500-nm or 510-550-nm. The result showed that GFP had been introduced in nearly 100% nerve cells that were MAP2-positive (Figure 15).

#### <Introduction into normal human cells>

Normal human smooth-muscle cells, normal human hepatic cells, and normal human pulmonary capillary endothelial cells (Cell Systems) were purchased from DAINIPPON PHARMACEUTICAL and were cultured with SFM CS-C medium kit (Cell Systems) at 37°C under 5% CO<sub>2</sub> gas.

Human normal cells, such as normal human smooth-muscle cells (Figure 15, Muscle), normal human hepatic cells (Figure 15, Liver), and normal human pulmonary capillary endothelial cells (Figure 15, Lung), were infected with F-deficient SeV vector (m.o.i=5), and then the expression of GFP was observed. It was verified that the introduction efficiency was nearly 100% and the GFP gene was expressed at very high levels in all the cells (Figure 15).

#### <Introduction into mouse primary bone marrow cells>

Further, an experiment was conducted, in which mouse primary bone marrow cells were separated by utilizing lineage markers and were infected with F-deficient SeV vector. First, 5-fluorouracil (5-FU, Wako Pure Chemical Industries) was given to C57BL mouse (6-week old male) at a dose of 150 mg/kg by intraperitoneal injection (IP injection); 2 days after the administration, bone marrow cells were collected from the thighbone. The mononuclear cells were separated by density gradient centrifugation using Lympholyte-M (Cedarlane). A mixture ( $3 \times 10^7$ ) of Streptavidin-magnetic beads (Pharmingen; Funakoshi), which had been coated with biotin-labeled anti-CD45R

(B220), anti-Ly6G (Gr-1), anti-Ly-76 (TER-119), anti-1 (Thy1.2), and anti-Mac-1, were added to the mononuclear cells ( $3 \times 10^6$  cells), and the resulting mixture was allowed to react at  $4^\circ\text{C}$  for 1 hour; a fraction, from which  $\text{Lin}^+$  cells had been removed by a magnet, was recovered ( $\text{Lin}^-$  cells) (Erlich, S. et al., Blood 1999. 93 (1), 80-86). SeV of  $2 \times 10^7$  HAU/ml was added to  $4 \times 10^5$  cells of  $\text{Lin}^-$  cell, and further recombinant rat SCF (100 ng/ml, BRL) and recombinant human IL-6 (100 U/ml) were added thereto. In addition, F-deficient SeV of  $4 \times 10^7$  CIU/ml was added to  $8 \times 10^5$  of total bone marrow cells, and GFP-SeV of  $5 \times 10^7$  CIU/ml was added to  $1 \times 10^6$  cells. GFP-SeV was prepared by inserting a PCR-amplified NotI fragment, which contains the green fluorescence protein (GFP) gene (the length of the structural gene is 717 bp) to which a transcription initiation (R1), a termination (R2) signal and an intervening (IG) sequence are added, at the restriction enzyme NotI-cleavage site of SeV transcription unit pSeV18+b(+) (Hasan, M. et al, J. Gen. Virol., 1997, 78:2813-2820). The reconstitution of viruses comprising the GFP gene was performed according to a known method (Genes Cells, 1996, 1: 569-579), using LLC-MK2 cells and embryonated egg, and then the viruses comprising the gene of interest were recovered. After a 48-hour culture following the infection with GFP-SeV, the cells were divided into two groups; one of them was allowed to react to phycoerythrin(PE)-labeled anti-CD117 (c-kit; Pharmingen) for 1 hour; the other was a control group. The cells were washed 3 times with PBS then were analyzed in a flow cytometer (EPICS Elite ESP; Coulter, Miami, FL).

The result showed that F-deficient SeV vector was also infected to bone marrow cells enriched by anti-c-kit antibody that has been utilized as a marker for blood primitive stem cells and the expression of the GFP gene was observed (Figure 16). The presence of infective particles in the culture supernatant was confirmed by determining the presence of GFP-expressing cells three days after the addition of cell culture supernatant treated with trypsin to LLC-MK2 cells. It was clarified that none of these cells released infective virus particles.

35

[Example 6] Vector administration into rat cerebral ventricle



Rats (F334/Du Crj, 6 week old, female, Charles River) were anesthetized by intraperitoneal injection of Nembutal sodium solution (Dainabot) diluted 10 folds (5 mg/ml) with physiological saline (Otsuka Pharmaceutical Co., Ltd.). Virus was administrated using  
 5 brain stereotaxic apparatus for small animals (DAVID KOPF). 20  $\mu$ l ( $10^8$  CIU) were injected at the point 5.2 mm toward bregma from interaural line, 2.0 mm toward right ear from lambda, 2.4 mm beneath the brain surface, using 30G exchangeable needles (Hamilton). A high level expression of GFP protein was observed in ventricle ependymal  
 10 cells (Figure 17). Furthermore, in the case of F deficient SeV vector, the expression of GFP protein was observed only in ependymal cells or nerve cells around the injection site, which come into contact with the virus, and no lesion was found in this region. Abnormality in behavior or changes in body weight were not observed in the  
 15 administered rats until dissection. After dissection, no lesion was found in the brain or in any of the tissues and organs analyzed, such as liver, lung, kidney, heart, spleen, stomach, intestine, and so forth.

20 [Example 7] Formation of F-less virus particles from F deficient SeV genome  
 <1>

F non-expressing LLC-MK2 cells and F expressing LLC-MK2 cells (LC-MK2/F7) were infected with F deficient SeV virus and cultured  
 25 with (+) and without (-) trypsin. The result of HA assay of cell culture supernatant after 3 days is shown in Figure 18A. The culture supernatants were inoculated to embryonated chicken eggs, and the result of HA assay of chorioallantoic fluids after a 2 day-culture is shown in Figure 18B. "C" on top of panel indicates PBS used as  
 30 the control group. The numbers indicated under "Dilution" indicates the dilution fold of the virus solution. Further, HA-positive chorioallantoic fluids in embryonated chicken eggs (lanes 11 and 12) was reinoculated into embryonated chicken eggs, and after culturing for two days, the chorioallantoic fluid was examined with HA assay  
 35 (Figure 19C). As a result, F non-expressing cells or embryonated chicken eggs infected with F deficient SeV virus were found to be

HA-positive. However, viruses had not propagated after re-inoculation to embryonated chicken eggs, proving that the HA-positive virus solution does not have secondary infectivity.

<2>

5 The non-infectious virus solution amplified in F non-expressing cells was examined for the existence of virus particles. Northern blot analysis was performed for total RNA prepared from the culture supernatant of F expressing cells, HA-positive, non-infectious chorioallantoic fluid, and wildtype SeV by QIAamp viral RNA mini kit  
10 (QIAGEN), using the F gene and HN gene as probes. As a result, bands were detected for RNA derived from chorioallantoic fluid or virus in culture supernatant of F expressing cells when the HN gene was used as the probe, whereas no bands were detected when using the F gene probe (Figure 10). It was proven that the HA-positive,  
15 non-infectious fluid has non-infectious virus-like particles with an F deficient genome. Further, analysis of the HA-positive, non-infectious virus solution by an immunoelectron microscopy revealed the existence of virus particles, and the envelope of virion reacted to the antibody recognizing gold colloid-labeled HN protein,  
20 but not to the antibody recognizing gold colloid-labeled F protein (Figure 20). This result showed the existence of F-less virions, proving that the virus can be formed as a virion with HN protein alone, even without the existence of the F protein. It has been shown that SeV virion can form with F alone (Leyer, S. et al., J Gen. Virol 79,  
25 683-687 (1998)), and the present result proved for the first time that SeV virion can be formed with HN protein alone. Thus, the fact that F-less virions can be transiently produced in bulk in embryonated chicken eggs shows that virions packaging SeV F deficient RNP can be produced in bulk.

30 <3>

As described above, F-less virions transiently amplified in embryonated chicken eggs are not at all infective towards cells infected by the Sendai virus. To confirm that functional RNP structures are packaged in envelopes, F expressing cells and  
35 non-expressing cells were, mixed with cationic liposome (DOSPER, Boehringer mannheim) and transfected by incubation for 15 minutes

at room temperature. As a result, GFP-expressing cells were not observed at all when the cells are not mixed with the cationic liposome, whereas all cells expressed GFP when mixed with cationic liposome. In F non-expressing cells, GFP expression was seen only in individual cells and did not extend to adjacent cells, whereas in F expressing cells, GFP-expressing cells extended to form colonies (Figure 21). Therefore, it became clear that non-infectious virions transiently amplified in embryonated chicken eggs could express a gene when they are introduced into cells by methods such as transfection.

[Example 8] Reconstitution and amplification of the virus from FHN-deficient SeV genome

<Construction of FHN-deficient genomic cDNA>

To construct FHN-deficient SeV genomic cDNA (pSeV18<sup>+</sup>/ΔFHN), pUC18/KS was first digested with EcoRI to construct pUC18/Eco, and then whole sequence from start codon of F gene to stop codon of HN gene (4866-8419) was deleted, then it was ligated at BsiWI site (cgtagc). After the sequence of FHN deleted region was confirmed by base sequencing, EcoRI fragment (4057 bp) was recovered from gels to substitute for EcoRI fragment of pUC18/KS to accomplish the construction. A KpnI/SphI fragment (14673 bp) comprising the FHN deleted region was recovered from gels to substitute for KpnI/SphI fragment of pSeV18<sup>+</sup> to obtain plasmid pSeV18<sup>+</sup>/ΔFHN.

On the other hand, the construction of FHN-deficient SeV cDNA introduced with GFP was accomplished as follows. SalI/XhoI fragment (7842 bp) was recovered from pSeV18<sup>+</sup>/ΔFHN, and cloned into pGEM11Z (Promega). The resultant plasmid was named as pGEM11Z/SXdFHN. To the FHN-deficient site of pGEM11Z/SXdFHN, PCR product with BsiXI sites at both ends of ATG-TAA (846 bp) of d2EGFP (Clontech) was ligated by digesting with BsiXI enzyme. The resultant plasmid was named as pSeV18<sup>+</sup>/ΔFHN-d2GFP.

<Establishment of FHN-deficient, protein co-expressing cell line>

The plasmid expressing F gene is identical to the one used for establishment of F deficient, protein co-expressing cell line, and plasmid expressing HN gene was similarly constructed, and the fragment comprising ORF of HN was inserted to unique SwaI site of pCALNdLw

(Arai et al., described above) to obtain plasmid named pCALNdLw/HN.

LLC-MK2 cells were mixed with same amount or different ratio of pCALNdLw/F and pCALNdLw/HN, to introduce genes using mammalian transfection kit (Stratagene), according to the manufacture's protocol. Cells were cloned after a three week-selection with G418. Drug resistant clones obtained were infected with a recombinant adenovirus (Ade/Cre, Saito et al., described above) (moi=10), which expresses Cre DNA recombinase. Then the cells were collected 3 days after inducing expression of F and HN protein after washing 3 times with PBS(-), and they were probed with monoclonal IgG of anti-SeV F protein and anti-SeV HN protein by using Western blotting method (Figure 22).

#### <Construction of pGEM/FHN>

F and HN fragments used for the construction of pCALNdLw/F and pCALNdLw/HN were cloned into pGEM4Z and pGEM3Z (Promega) to obtain pGEM4Z/F and pGEM3Z/HN, respectively. A fragment obtained by PvuII digestion of the region comprising T7 promoter and HN of pGEM3Z/HN was recovered, and ligated into the blunted site cut at the SacI unique site at the downstream of F gene of pGEM4Z/F. F and HN proteins were confirmed by Western blotting using anti-F or anti-HN monoclonal antibodies to be expressed simultaneously when they were aligned in the same direction.

#### <Reconstitution of FHN-deficient virus>

The reconstitution of FHN-deficient viruses (P0) was done in two ways. One was using the RNP transfection method as used in the reconstitution of F deficient virus, and the other was using T7 to supply co-expressing plasmids. Namely, under the regulation of T7 promoter, plasmids expressing F and HN proteins were constructed separately, and using those plasmids F and HN proteins were supplied for the reconstitution. In both methods, reconstituted viruses were amplified by FHN coexpressing cells. FHN-deficient, GFP-expressing SeV cDNA (pSeV18<sup>+</sup>/ΔFHN-d2GFP), pGEM/NP, pGEM/P, pGEM/L, and pGEM/FHN were mixed in the ratio of 12 μg/10 cm dish, 4 μg/10 cm dish, 2 μg/10 cm dish, 4 μg/10 cm dish, and 4 μg/10 cm dish (final total volume, 3 ml/10 cm dish) for gene introduction into LLC-MK2 cells in the same way as F deficient SeV reconstitution described above. Three hours

after the gene introduction, media was changed to MEM containing AraC (40  $\mu$ g/ml, SIGMA) and trypsin (7.5  $\mu$ g/ml, GIBCO), and cultured further for 3 days. Observation was carried out by fluorescence stereoscopic microscope 2 days after gene introduction. The effect of pGEM/FHN addition was analyzed, and the virus formation was confirmed by the spread of GFP-expressing cells. As a result, a spread of GFP-expressing cells was observed when pGEM/FHN was added at reconstitution, whereas the spread was not observed when pGEM/FHN was not added, and the GFP expression was observed only in a single cell (Figure 23). It is demonstrated that the addition at FHN protein reconstitution caused virus virion formation. On the other hand, in the case of RNP transfection, virus recovery was successfully accomplished in FHN expressing cells of P1, as in the case of F deficiency (Figure 24, upper panel).

Virus amplification was confirmed after infection of FHN-deficient virus solution to cells induced to express FHN protein 6 hours or more after Ade/Cre infection (Figure 24, lower panel).

Solution of viruses reconstituted from FHN-deficient GFP-expressing SeV cDNA was infected to LLC-MK2, LLC-MK2/F, LLC-MK2/HN and LLC-MK2/FHN cells, and cultured with or without the addition of trypsin. After 3 days of culture, spread of GFP protein expressing cells was analyzed. As a result, spread of GFP was observed only in LLC-MK2/FHN, confirming that the virus solution can be amplified specifically by FHN co-expression and in a trypsin dependent manner (Figure 25).

To confirm FHN-deficient viral genome, culture supernatant recovered from LLC-MK2/FHN cells was centrifuged, and RNA was extracted using QIAamp Viral RNA mini kit (QIAGEN), according to manufacturer's protocol. The RNA was used for template synthesis of RT-PCR using Superscript Preamplification System for first Strand Synthesis (GIBCO BRL), and PCR was performed using TAKARA Z-Taq (Takara). F-deficient virus was used as a control group. PCR primer sets were selected as combination of M gene and GFP gene, or combination of M gene and L gene (for combination of M gene and GFP gene (M-GFP), forward: 5'-atcagagacctgcgacaatgc / SEQ ID NO: 13, reverse: 5'-aagtcgtgctgcttcattgtgg / SEQ ID NO: 14; for combination

of M gene and L gene (M-L), forward: 5'-gaaaaacttagggataaagtccc /  
SEQ ID NO: 15, reverse: 5'-gttatctccgggatggtgc / SEQ ID NO: 16). As  
a result, specific bands were obtained for both F-deficient and  
FHN-deficient viruses at RT conditions when using M and GFP genes  
5 as primers. In the case of using M and L genes as primers, the bands  
with given size comprising GFP were detected for FHN deficient sample,  
and lengthened bands with the size comprising HN gene were detected  
for F deficient one. Thus, FHN deficiency in genome structure was  
proven (Figure 26).

10 On the other hand, FHN-deficient virus was infected to F  
expressing cells similarly as when using the F-deficient virus, and  
culture supernatant was recovered after 4 days to perform infection  
experiment toward LLC-MK2, LLC-MK2/F, and LLC-MK2/FHN. As a result,  
GFP expression cell was not observed in any infected cell, showing  
15 that the virus has no infectiousness to these cells. However, it has  
been already reported that F protein alone is enough to form virus  
particles (Leyer, S. et al, J. Gen. Virol.79, 683-687 (1998)) and  
that asialoglycoprotein receptor (ASG-R) mediates specific infection  
to hepatocytes (Spiegel et al., J. Virol 72, 5296-5302, 1998). Thus,  
20 virions comprising FHN-deficient RNA genome, with virus envelope  
configured with only F protein may be released to culture supernatant  
of F expressing cells. Therefore, culture supernatant of F  
expressing cells infected with FHN-deficient virus was recovered,  
and after centrifugation, RNA was extracted as described above and  
25 analyzed by RT-PCR by the method described above. As a result, the  
existence of RNA comprising FHN-deficient genome was proved as shown  
in Figure 27.

Western blotting analysis of virus virion turned into  
pseudotype with VSV-G clearly shows that F and HN proteins are not  
30 expressed. It could be said that herein, the production system of  
FHN-deficient virus virions was established.

Moreover, virions released from F protein expressing cells were  
overlaid on FHN expressing or non-expressing LLC-MK2 cells with or  
without mixing with a cationic liposome (50  $\mu$ l DOSPER/500  $\mu$ l/well).  
35 As a result, spread of GFP-expressing cells was observed when overlaid  
as mixture with DOSPER, while HN-less virion only has no

infectiousness at all, not showing GFP-expressing cells, as was seen in the case of F-less particles described above. In FHN non-expressing cells GFP expressing cell was observed, but no evidence of virus re-formation and spread was found.

5        These virus-like particles recovered from F expressing cells can infect cells continuously expressing ASG-R gene, ASG-R non-expressing cells, or hepatocytes, and whether the infection is liver-specific or ASG-R specific can be examined by the method of Spiegel et al.

10        [Example 9] Application of deficient genome RNA virus vector

1. F-deficient RNP amplified in the system described above is enclosed by the F-less virus envelope. The envelope can be introduced into cells by adding any desired cell-introducing capability to the  
15 envelope by chemical modification methods and such, or by gene introducing reagents or gene guns or the like (RNP transfection, or RNP injection), and the recombinant RNA genome can replicate and produce proteins autonomously and continuously in the cells.

2. A vector capable of specific targeting can be produced, when  
20 intracellular domain of HN is left as-is, and the extracellular domain of HN is fused with ligands capable of targeting other receptors in a specific manner, and recombinant gene capable of producing chimeric protein is incorporated into viral-genome. In addition, the vector can be prepared in cells producing the recombinant protein. These  
25 vectors can be applicable to gene therapy, as vaccines, or such.

3. Since the reconstitution of SeV virus deficient in both FHN has been successfully accomplished, targeting vector can be produced by introducing targeting-capable envelope chimeric protein gene into FHN deletion site instead of the GFP gene, reconstituting it by the  
30 same method as in the case of FHN-deficient vector, amplifying the resultant once in FHN-expressing cells, infecting the resultant to non-expressing cells, and recovering virions formed with only the targeting-capable chimeric envelope protein transcribed from the viral-genome.

35        4. A mini-genome of Sendai virus and a virion formed with only F protein packaging mini-genome by introducing NP, P, L and F gene to

cells have been reported (Leyer et al., J Gen. Virol 79, 683-687, 1998). A vector in which murine leukemia virus is turned into pseudo-type by Sendai F protein has also been reported (Spiegel et al., J. Virol 72, 5296-5302, 1998). Also reported so far is the specific targeting of trypsin-cleaved F-protein to hepatocytes mediated by ASG-R (Bitzer et al., J. Virol. 71, 5481-5486, 1997). The systems in former reports are transient particle-forming systems, which make it difficult to continuously recover vector particles. Although Spiegel et al. has reported retrovirus vector turned into pseudo-type by Sendai F protein, this method carries intrinsic problems like the retrovirus being able to introduce genes to only mitotic cells. The virus particles recovered in the present invention with a FHN co-deficient SeV viral-genome and only the F protein as the envelope protein are efficient RNA vectors capable of autonomous replication in the cytoplasm irrespective of cell mitosis. They are novel virus particles, and is a practical system facilitating mass production.

[Example 10] Virus reconstitution and amplification from FHN-deficient SeV genome

The techniques of reconstitution of infectious virus particles from cDNA that cloned the viral genome has been established for many single-strand minus strand RNA viruses such as the Sendai virus, measles virus.

In most of the systems, reconstitution is carried out by introducing plasmids introduced with cDNA, NP, P, and L genes at the downstream of T7 promoter into cells and expressing cDNA and each gene using T7 polymerase. To supply T7 polymerase, recombinant vaccinia virus expressing T7 polymerase is mainly used.

T7 expressing vaccinia virus can express T7 polymerase efficiently in most cells. Although, because of vaccinia virus-induced cytotoxicity, infected cells can live for only 2 or 3 days. In most cases, rifampicin is used as an anti-vaccinia reagent. In the system of Kato et al. (Kato, A. et al., Genes cells 1, 569-579 (1996)), AraC was used together with rifampicin for inhibiting vaccinia virus growth to a minimum level, and efficient reconstitution of Sendai virus.



However, the reconstitution efficiency of minus strand RNA virus represented by Sendai virus is several particles or less in  $1 \times 10^5$  cells, far lower than other viruses such as retroviruses. Cytotoxicity due to the vaccinia virus and the complex reconstitution process (transcribed and translated protein separately attaches to bare RNA to form RNP-like structure, and after that, transcription and translation occurs by a polymerase) can be given as reasons for this low reconstitution efficiency.

In addition to the vaccinia virus, an adeno virus system was examined as a means for supplying T7 polymerase, but no good result was obtained. Vaccinia virus encodes RNA capping enzyme functioning in cytoplasm as the enzyme of itself in addition to T7 polymerase and it is thought that the enzyme enhances the translational efficiency by capping the RNA transcribed by T7 promoter in the cytoplasm. The present invention tried to enhance the reconstitution efficiency of Sendai virus by treating vaccinia virus with Psoralen-Long-Wave-UV method to avoid cytotoxicity due to the vaccinia virus.

By DNA cross-linking with Psoralen and long-wave ultraviolet light, the state in which the replication of virus with DNA genome is inhibited, without effecting early gene expression in particular, can be obtained. The notable effect seen by inactivation of the virus in the system may be attributed to that vaccinia virus having a long genome (Tsung, K. et al., J Virol 70, 165-171 (1996)).

In the case of wildtype virus that can propagate autonomously, even a single particle of virus formed by reconstitution makes it possible for Sendai virus to be propagated by inoculating transfected cells to embryonated chicken eggs. Therefore, one does not have to consider of the efficiency of reconstitution and the residual vaccinia virus seriously.

However, in the case of reconstitution of various mutant viruses for researching viral replication, particle formation mechanism, and so on, one may be obligated to use cell lines expressing a protein derived from virus and such, not embryonated chicken eggs, for propagation of the virus. Further, it may greatly possible that the mutant virus or deficient virus propagates markedly slower than the

wild type virus.

To propagate Sendai virus with such mutations, transfected cells should be overlaid onto cells of the next generation and cultured for a long period. In such cases, the reconstitution efficiency and residual titer of vaccinia virus may be problematic. In the present method, titer of surviving vaccinia virus was successfully decreased while increasing reconstitution efficiency.

Using the present method, a mutant virus that could have not been ever obtained in the former system using a non-treated vaccinia virus was successfully obtained by reconstitution (F, FHN-deficient virus). The present system would be a great tool for the reconstitution of a mutant virus, which would be done more in the future. Therefore, the present inventors examined the amount of Psoralen and ultraviolet light (UV), and the conditions of vaccinia virus inactivation.

#### <Experiment>

First, Psoralen concentration was tested with a fixed irradiation time of 2 min. Inactivation was tested by measuring the titer of vaccinia virus by plaque formation, and by measuring T7 polymerase activity by pGEM-luci plasmid under the control of T7 promoter and mini-genome of Sendai virus. The measurement of T7 polymerase activity of mini-genome of Sendai virus is a system in which cells are transfected concomitantly with plasmid of mini-genome of Sendai virus and pGEM/NP, pGEM/P, and pGEM/L plasmids, which express NP-, P-, and L-protein of Sendai virus by T7, to examine transcription of the RNA encoding luciferase enzyme protein by RNA polymerase of Sendai virus after the formation of ribonucleoprotein complex.

After the 2 min UV irradiation, decrease in titer of vaccinia virus depending on psoralen concentration was seen. However, T7 polymerase activity was unchanged for a Psoralen concentration up to 0, 0.3, and 1  $\mu\text{g/ml}$ , but decreased approximately to one tenth at 10  $\mu\text{g/ml}$  (Figure 28).

Furthermore, by fixing Psoralen concentration to 0.3  $\mu\text{g/ml}$ , UV irradiation time was examined. In accordance with the increase of irradiation time, the titer of vaccinia virus was decreased, although

no effect on T7 polymerase activity was found up to a 30 min irradiation. In this case, under the conditions of 0.3  $\mu\text{g/ml}$  and 30 min irradiation, titer could be decreased down to 1/1000 without affecting T7 polymerase activity (Figure 29).

5           However, in vaccinia virus with a decreased titer of 1/1000, CPE 24 hours after infection at  $\text{moi}=2$  calibrated to pretreatment titer ( $\text{moi}=0.002$  as residual titer after treatment) was not different from that of non-treated virus infected at  $\text{moi}=2$  (Figure 30).

10           Using vaccinia virus treated under the conditions described above, the efficiency of reconstitution of Sendai virus was examined. Reconstitution was carried out by the procedure described below, modifying the method of Kato et al. mentioned above. LLC-MK2 cells were seeded onto 6-well microplates at  $3 \times 10^5$  cells/well, and after an overnight culture, vaccinia virus was diluted to the titer of  $6 \times 10^5$  pfu/100  $\mu\text{l}$  calibrated before PLWUV treatment, and infected to  
15           PBS-washed cells. One hour after infection, 100  $\mu\text{l}$  of OPTI-MEM added with 1, 0.5, 1, and 4  $\mu\text{g}$  of plasmid pGEM-NP, P, L, and cDNA, respectively, was further added with 10  $\mu\text{l}$  Superfect (QIAGEN) and left standing for 15 min at room temperature, and after adding 1 ml OPTI-MEM (GIBCO)  
20           (containing Rif. and AraC), was overlaid onto the cells.

          Two, three and four days after transfection, cells were recovered, centrifuged, and suspended in 300  $\mu\text{l}$ /well of PBS. 100  $\mu\text{l}$  of cell containing solution made from the suspension itself, or by diluting the suspension by 10 or 100 folds, was inoculated to  
25           embryonated chicken eggs at day 10 following fertilization, 4 eggs for each dilution ( $1 \times 10^5$ ,  $1 \times 10^4$ , and  $1 \times 10^3$  cells, respectively). After 3 days, allantoic fluid was recovered from the eggs and the reconstitution of virus was examined by HA test (Table 1). Eggs with HA activity was scored as 1 point, 10 points and 100 points for eggs  
30           inoculated with  $1 \times 10^5$ ,  $1 \times 10^4$ , and  $1 \times 10^3$  cells, respectively, to calculate the Reconstitution Score (Figure 31). The formula is as shown in Table 1.

Table 1. Effect of the duration of UV treatment of vaccinia virus on reconstitution efficiency of Sendai virus

The number of inoculated cells	Score (a)	The number of HA positive eggs (b)											
		2d						3d					
		0'	15'	20'	30'	0'	15'	20'	30'	0'	15'	20'	30'
$10^5$	1 (a1)	1	2	4	4	0	2	4	4	1	3	4	4
$10^4$	10 (a2)	0	1	3	2	0	2	3	4	0	0	4	0
$10^3$	100 (a3)	0	0	0	1	0	1	0	2	0	0	0	0
Reconsti- tution Score	(a1+a2+a3) x b	1	12	24	124	0	122	34	244	1	3	44	4

$$\text{Reconstitution Score} = (a1+a2+a3) \times b$$

Also, residual titers of vaccinia virus measured at 2, 3, and 4 days after transfection within cells were smaller in the treated group in proportion to the titer given before transfection (Figure 32).

By inactivating vaccinia virus by PLWUV, titer could be decreased down to 1/1000 without affecting T7 polymerase activity. However, CPE derived from vaccinia virus did not differ from that of non-treated virus with a 1000 fold higher titer as revealed by

microscopic observations.

Using vaccinia virus treated with the condition described above for reconstitution of Sendai virus, reconstitution efficiency increased from ten to hundred folds (Figure 31). At the same time, residual titer of vaccinia virus after transfection was not 5 pfu/10<sup>5</sup> cells or more. Thus, the survival of replicable vaccinia virus was kept at 0.005% or less.

[Example 11] Construction of pseudotype Sendai virus

<1> Preparation of helper cells in which VSV-G gene product is induced

Because VSV-G gene product has a cytotoxicity, stable transformant was created in LLC-MK2 cells using plasmid pCALNdLG (Arai T. et al., J. Virology 72 (1998) p1115-1121) in which VSV-G gene product can be induced by Cre recombinase. Introduction of plasmid into LLC-MK2 cells was accomplished by calcium phosphate method (CalPhosTMMammalian Transfection Kit, Clontech), according to accompanying manual.

Ten micrograms of plasmid pCALNdLG was introduced into LLC-MK2 cells grown to 60% confluency in a 10 cm culture dish. Cells were cultured for 24 hours with 10 ml MEM-FCS 10% medium in a 5% CO<sub>2</sub> incubator at 37°C. After 24 hours, cells were scraped off and suspended in 10 ml of medium, and then using five 10 cm culture dishes, 1, 2 and 2 dishes were seeded with 5 ml, 2 ml and 0.5 ml, respectively. Then they were cultured for 14 days in 10 ml MEM-FCS 10% medium containing 1200 µg/ml G418 (GIBCO-BRL) with a medium change on every other day to select stable transformants. Twenty-eight clones resistant to G418 grown in the culture were recovered using cloning rings. Each clone was expanded to confluency in a 10 cm culture dish.

For each clone, the expression of VSV-G was examined by Western blotting described below using anti-VSV-G monoclonal antibody, after infection with recombinant adenovirus AxCANCre containing Cre recombinase.

Each clone was grown in a 6 cm culture dish to confluency, and after that, adenovirus AxCANCre was infected at MOI=10 by the method of Saito et al. (see above), and cultured for 3 days. After removing the culture supernatant, the cells were washed with PBS, and detached

from the culture dish by adding 0.5 ml PBS containing 0.05% trypsin and 0.02% EDTA (ethylenediaminetetraacetic acid) and incubating at 37°C, 5 min. After suspending in 3 ml PBS, the cells were collected by centrifugation at 1500x g, 5 min. The cells obtained were resuspended in 2 ml PBS, and then centrifuged again at 1500x g, 5 min to collect cells.

The cells can be stored at -20°C, and can be used by thawing according to needs. The collected cells were lysed in 100 µl cell lysis solution (RIPA buffer, Boehringer Mannheim), and using whole protein of the cells ( $1 \times 10^5$  cells per lane) Western blotting was performed. Cell lysates were dissolved in SDS-polyacrylamide gel electrophoresis sample buffer (buffer comprising 6 mM Tris-HCl (pH6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol) and subjected as samples for electrophoresis after heating at 95°C, 5 min. The samples were separated by electrophoresis using SDS-polyacrylamide gel (Multigel 10/20, Daiichi Pure Chemicals Co., Ltd), and the separated protein was then transferred to transfer membrane (Immobilon-P TransferMembranes, Millipore) by semi-dry blotting method. Transfer was carried out using transfer membrane soaked with 100% methanol for 20 sec and with water for 1 hour, at a 1 mA/cm<sup>2</sup> constant current for 1 hour.

The transfer membrane was shaken in 40 ml of blocking solution (Block-Ace, Snow Brand Milk Products Co., Ltd.) for 1 hour, and washed once in PBS.

The transfer membrane and 5 ml anti-VSV-G antibody (clone P4D4, Sigma) diluted 1/1000 by PBS containing 10% blocking solution were sealed in a vinyl-bag and left to stand at 4°C.

The transfer membrane was soaked twice in 40 ml of PBS-0.1% Tween 20 for 5 min, and after the washing, soaked in PBS for 5 min for washing.

The transfer membrane and 5 ml of anti-mouse IgG antibody labeled with peroxidase (anti-mouse immunoglobulin, Amersham) diluted to 1/2500 in PBS containing 10% blocking solution were sealed in vinyl-bag and were shaken at room temperature for 1 hour.

After shaking, the transfer membrane was soaked twice in PBS-0.1% Tween 20 for 5 min, and after the washing, soaked in PBS for 5 min for washing.

The detection of proteins on the membrane crossreacting with anti-VSV-G antibody was carried out by the luminescence method (ECL Western blotting detection reagents, Amersham). The result is shown in Figure 33. Three clones showed AxCANCre infection specific VSV-G expression, confirming the establishment of LLC-MK2 cells in which VSV-G gene product can be induced.

One clone among the clones obtained, named as LLCG-L1, was subjected to flow cytometry analysis using anti-VSV antibody (Figure 34). As a result, reactivity with antibody specific to VSV-G gene induction was detected in LLCG-L1, confirming that VSV-G protein is expressed on the cell surface.

<2> Preparation of pseudotype Sendai virus comprising a genome deficient in the F gene using helper cells

Sendai virus comprising a genome deficient in F gene was infected to VSV-G gene expressing cells, and whether production of pseudotype virus with VSV-G as capsid can be seen or not was examined using F-deficient Sendai virus comprising GFP gene described in the examples above, and the expression of GFP gene as an index. As a result, in LLCG-L1 without infection of recombinant adenovirus AxCANCre comprising Cre recombinase, viral gene was introduced by F-deficient Sendai virus infection and GFP-expressing cells were detected, although the number of expressing cells was not increased. In VSV-G induced cells, chronological increase of GFP-expressing cells was found. When 1/5 of supernatants were further added to newly VSV-G induced cells, no gene introduction was seen in the former supernatant, while the increase of GFP-expressing cells as well as gene introduction were found in the latter supernatant. Also, in the case that supernatant from latter is added to LLCG-L1 cells without induction of VSV-G, gene was introduced, but increase of GFP-expressing cells was not seen. Taken together, virus propagation specific to VSV-G expressing cells was found, and pseudotype F-deficient virus formation with VSV-G was found.

<3> Evaluation of conditions for producing pseudotype Sendai virus with F gene-deficient genome

A certain amount of pseudotype Sendai viruses with F

gene-deficient genomes was infected changing the amount of AxCANCre infection (MOI=0, 1.25, 2.5, 5, and 10) and culture supernatant was recovered at day 7 or day 8. Then, the supernatant was infected to the cells before and after induction of VSV-G, and after 5 days, number of cells expressing GFP was compared to see the effect of amount of VSV-G gene expression. As a result, no virus production was found at MOI=0 and maximum production was found at MOI=10 (Figure 35). In addition, when time course of virus production was analyzed, the production level started to increase from day 5 or after, persisting to day 8 (Figure 36). The measurement of virus titer was accomplished by calculating the number of particles infected to cells in the virus solution (CIU), by counting GFP-expressing cells 5 days after infection of serially (10 fold each) diluted virus solutions to cells not yet induced with VSV-G. As a result, the maximal virus production was found to be  $5 \times 10^5$  CIU/ml.

#### <4> Effect of anti-VSV antibody on infectiousness of pseudotype Sendai virus with F gene-deficient genome

As to whether pseudotype Sendai virus with F gene-deficient genome obtained by using VSV-G expressing cells comprises VSV-G protein in the capsid, the neutralizing activity of whether infectiousness will be affected was evaluated using anti-VSV antibody. Virus solution and antibody were mixed and left standing at room temperature for 30 min, and then infected to LLCG-L1 cells without VSV-G induction. On day 5, gene-introducing capability was examined by the existence of GFP-expressing cells. As a result, perfect inhibition of infectiousness was seen by the anti-VSV antibody, whereas in Sendai virus with F gene-deficient genome having the original capsid, the inhibition was not seen (Figure 37). Therefore, it was clearly shown that the present virus obtained is a pseudotype Sendai virus comprising VSV-G protein in its capsid, in which infectiousness of the virus can be specifically inhibited by an antibody.

#### <5> Confirmation of pseudotype Sendai virus's possession of F-deficient genome

Western blotting analysis of cell extract of infected cells was carried out to examine if the present virus propagated in cells



expressing VSV-G gene is the F-deficient type. Western analysis was accomplished by the method described above. As the primary antibodies, anti-Sendai virus polyclonal antibody prepared from rabbit, anti-F protein monoclonal antibody prepared from mouse, and anti-HN protein monoclonal antibody prepared from mouse were used. As the secondary antibodies, anti-rabbit IgG antibody labeled with peroxidase in the case of anti-Sendai virus polyclonal antibody, and anti-mouse IgG antibody labeled with peroxidase in the case of anti-F protein monoclonal antibody and anti-HN protein monoclonal antibody, were used. As a result, F protein was not detected, whereas protein derived from Sendai virus and HN protein were detected, confirming it is F-deficient type.

<6> Preparation of pseudotype Sendai virus with F and HN gene-deficient genome by using helper cells

Whether the production of pseudotype virus with VSV-G in its capsid is observed after the infection of Sendai virus with F and HN gene-deficient genome to LLCG-L1 cells expressing VSV-G gene was analyzed using GFP gene expression as the indicator and F and HN gene-deficient Sendai virus comprising GFP gene described in examples above, by a similar method as described in examples above. As a result, virus propagation specific to VSV-G expressing cells was observed, and the production of F and HN deficient Sendai virus that is a pseudotype with VSV-G was observed (Figure 38). The measurement of virus titer was accomplished by calculating the number of particles infected to cells in the virus solution (CIU), by counting GFP-expressing cells 5 days after infection of serially (10 fold each) diluted virus solutions to cells not yet induced with VSV-G. As a result, the maximal virus production was  $1 \times 10^6$  CIU/ml.

<7> Confirmation of pseudotype Sendai virus's possession of F and HN deficient genome

Western blotting of proteins in cell extract of infected cells was carried out to analyze whether the present virus propagated in VSV-G expressing cells are the F and HN deficient type. As a result, F and HN proteins were not detected, whereas proteins derived from Sendai virus were detected, confirming that it is F and HN deficient type (Figure 39).

[Example 12] Analysis of virus reconstitution method  
 <Conventional method>

LLC-MK2 cells were seeded onto 100 mm culture dishes at  $5 \times 10^6$  cells/dish. After a 24 hour culture, the cells were washed once with MEM medium without serum, and then infected with recombinant vaccinia virus expressing T7 RNA polymerase (Fuerst, T.R. et al., Proc. Natl. Acad. Sci. USA 83, 8122-8126 1986) (vTF7-3) at room temperature for 1 hour ( $\text{moi}=2$ ) ( $\text{moi}=2$  to 3, preferably  $\text{moi}=2$  is used). The virus used herein, was pretreated with  $3 \mu\text{g/ml}$  psoralen and long-wave ultraviolet light (365 nm) for 5 min. Plasmids pSev18<sup>+</sup>/ΔF-GFP, pGEM/NP, pGEM/P, and pGEM/L (Kato, A. et al., Genes cells 1, 569-579 (1996)) were suspended in Opti-MEM medium (GIBCO) at ratio of  $12 \mu\text{g}$ ,  $4 \mu\text{g}$ ,  $2 \mu\text{g}$ , and  $4 \mu\text{g/dish}$ , respectively. Then, SuperFect transfection reagent (1  $\mu\text{g}$  DNA/5  $\mu\text{l}$ , QIAGEN) was added and left to stand at room temperature for 15 min and 3 ml Opti-MEM medium containing 3% FBS was added. Thereafter, the cells were washed twice with MEM medium without serum, and DNA-SuperFect mixture was added. After a 3 hr culture, cells were washed twice with MEM medium without serum, and cultured 70 hours in MEM medium containing  $40 \mu\text{g/ml}$  cytosine  $\beta$ -D-arabinofuranoside (AraC, Sigma). Cells and culture supernatant were collected as P0-d3 samples. Pellets of P0-d3 were suspended in Opti-MEM medium ( $10^7$  cells/ml). They were freeze-thawed three times and then mixed with lipofection reagent DOSPER (Boehringer Mannheim) ( $10^6$  cells/25  $\mu\text{l}$  DOSPER) and left to stand at room temperature for 15 min. Then, F expressing LLC-MK2/F7 cells were transfected with the mixture ( $10^6$  cells/well in 24-well plate) and cultured with MEM medium without serum (containing  $40 \mu\text{g/ml}$  AraC and  $7.5 \mu\text{g/ml}$  trypsin). Culture supernatants were recovered on day 3 and day 7 and were designated as P1-d3 and P1-d7 samples.

<Envelope plasmid + F expressing cells overlaying method>

Transfection was carried out similarly as described above, except that  $4 \mu\text{g/dish}$  envelope plasmid pGEM/FHN was added. After a 3 hr culture, cells were washed twice with MEM medium without serum, and cultured 48 hours in MEM medium containing  $40 \mu\text{g/ml}$  cytosine  $\beta$ -D-arabinofuranoside (AraC, Sigma) and  $7.5 \mu\text{g/ml}$  trypsin. After

removing the culture supernatant, cells were overlaid with 5 ml cell suspension solution of a 100 mm dish of F expressing LLC-MK2/F7 cells suspended with MEM medium without serum (containing 40  $\mu\text{g/ml}$  AraC and 7.5  $\mu\text{g/ml}$  trypsin). After a 48 hr culture, cells and supernatants were recovered and designated as P0-d4 samples. Pellets of P0-d4 samples were suspended in Opti-MEM medium ( $2 \times 10^7$  cells/ml) and freeze-thawed three times. Then F expressing LLC-MK2/F7 cells were overlaid with the suspension ( $2 \times 10^6$  cells/well, 24-well plate) and cultured in MEM medium without serum (containing 40  $\mu\text{g/ml}$  AraC and 7.5  $\mu\text{g/ml}$  trypsin). Culture supernatants were recovered on day 3 and day 7 of the culture, designated as P1-d3 and P1-d7 samples, respectively. As a control, experiment was carried out using the same method as described above, but without overlaying and adding only the envelope plasmid.

<CIU (Cell Infectious Units) measurement by counting GFP-expressing cells (GFP-CIU)>

LLC-MK2 cells were seeded onto a 12-well plate at  $2 \times 10^5$  cells/well, and after 24 hr culture the wells were washed once with MEM medium without serum. Then, the cells were infected with 100  $\mu\text{l/well}$  of appropriately diluted samples described above (P0-d3 or P0-d4, P1-d3, and P1-d7), in which the samples were diluted as containing 10 to 100 positive cells in  $10 \text{ cm}^2$ . After 15 min, 1 ml/well of serum-free MEM medium was added, and after a further 24 hr culture, cells were observed under fluorescence microscopy to count GFP-expressing cells.

<Measurement of CIU (Cell Infectious Units)>

LLC-MK2 cells were seeded onto a 12-well plate at  $2 \times 10^5$  cells/dish and after a 24 hr culture, cells were washed once with MEM medium without serum. Then, the cells were infected with 100  $\mu\text{l/well}$  of samples described above, in which the virus vector contained is designated as SeV/ $\Delta\text{F}$ -GFP. After 15 min, 1 ml/well of MEM medium without serum was added and cultured for a further 24 hours. After the culture, cells were washed with PBS (-) three times and were dried up by leaving standing at room temperature for approximately 10 min to 15 min. To fix cells, 1 ml/well acetone was added and immediately removed, and then the cells were dried up again

by leaving to stand at room temperature for approximately 10 min to 15 min. 300  $\mu$ l/well of anti-SeV polyclonal antibody (DN-1) prepared from rabbit, 100-fold diluted with PBS (-) was added to cells were and incubated for 45 min at 37°C. Then, they were washed three times  
 5 with PBS (-) and 300  $\mu$ l/well of anti-rabbit IgG (H+L) fluorescence-labeled second antibody (Alexa™568, Molecular Probes), 200-fold diluted with PBS (-) was added and incubated for 45 min at 37°C. After washing with PBS (-) three times, the cells were observed  
 10 under fluorescence microscopy (Emission: 560 nm, Absorption: 645 nm filters, Leica) to find florescent cells (Figure 40).

As controls, samples described above (SeV/ $\Delta$ F-GFP) were infected at 100  $\mu$ l/well, and after 15 min 1 ml/well of MEM without serum was added, and after a 24 hr culture, cells were observed under  
 15 fluorescence microscopy (Emission: 360 nm, Absorption: 470 nm filters, Leica) to find GFP-expressing cells, without the process after the culture.

[Example 13] Evaluation of the most suitable PLWUV (Psoralen and Long-Wave UV light) treatment conditions for vaccinia virus (vTF7-3)  
 20 for increasing reconstitution efficiency of deficient-type Sendai virus vector

LLC-MK2 cells were seeded onto 100 mm culture dishes at  $5 \times 10^6$  cells/dish, and after a 24 hr culture, the cells were washed once with MEM medium without serum. Then, the cells were infected with  
 25 recombinant vaccinia virus (vTF7-3) (Fuerst, T.R. et al., Proc. Natl. Acad. Sci. USA 83, 8122-8126 (1986)) expressing T7 RNA polymerase at room temperature for 1 hour (moi=2) (moi=2 to 3, preferably moi=2 is used). The virus used herein, was pretreated with 0.3 to 3  $\mu$ g/ml psoralen and long-wave ultraviolet light (365 nm) for 2 to 20 min.  
 30 Plasmids pSeV18<sup>+</sup>/ $\Delta$ F-GFP, pGEM/NP, pGEM/P, and pGEM/L (Kato, A. et al., Genes cells 1, 569-579 (1996)) were suspended in Opti-MEM medium (GIBCO) at ratio of 12  $\mu$ g, 4  $\mu$ g, 2  $\mu$ g, and 4  $\mu$ g/dish, respectively. Then, SuperFect transfection reagent (1  $\mu$ g DNA/5  $\mu$ l, QIAGEN) was added and left to stand at room temperature for 15 min and 3 ml Opti-MEM  
 35 medium containing 3% FBS was added. Thereafter, the cells were washed twice with MEM medium without serum, and then DNA-SuperFect mixture

was added. After a 3 hr culture, cells were washed twice with MEM medium without serum, and cultured 48 hours in MEM medium containing 40  $\mu\text{g/ml}$  cytosine  $\beta$ -D-arabinofuranoside (AraC, Sigma). Approximately 1/20 of field of view in 100 mm culture dish was observed by a fluorescence microscope and GFP-expressing cells were counted. To test the inactivation of vaccinia virus (vTF7-3), titer measurement by plaque formation (Yoshiyuki Nagai et al., virus experiment protocols, p291-296, 1995) was carried out.

Further, fixing the timing of recovery after transfection to day 3, psoralen and UV irradiation time were examined. Using vaccinia virus (vTF7-3) treated with each PLWUV treatment, reconstitution efficiency of Sendai virus was examined. Reconstitution was carried out by modifying the method of Kato et al., namely by the procedure described below. LLC-MK2 cells were seeded onto a 6-well microplate at  $5 \times 10^5$  cells/well, and after an overnight culture (cells were considered to grow to  $1 \times 10^6$  cells/well), PBS washed cells were infected with diluted vaccinia virus (vTF7-3) at  $2 \times 10^6$  pfu/100  $\mu\text{l}$  calibrated by titer before PLWUV treatment. After a 1 hour infection, 50  $\mu\text{l}$  of Opti-MEM medium (GIBCO) was added with 1, 0.5, 1, and 4  $\mu\text{g}$  of plasmid pGEM/NP, pGEM/P, pGEM/L, and additional type SeV cDNA (pSeV18<sup>+</sup>b (+)) (Hasan, M. K. et al., J. General Virology 78: 2813-2820, 1997), respectively. 10  $\mu\text{l}$  SuperFect (QIAGEN) was further added and left to stand at room temperature for 15 min. Then, 1 ml of Opti-MEM (containing 40  $\mu\text{g/ml}$  AraC) was added and overlaid onto the cells. Cells were recovered 3 days after transfection, then centrifuged and suspended in 100  $\mu\text{l/well}$  PBS. The suspension was diluted 10, 100, and 1000-fold and 100  $\mu\text{l}$  of resultant cell solution was inoculated into embryonated chicken eggs 10 days after fertilization, using 3 eggs for each dilution ( $1 \times 10^5$ ,  $1 \times 10^4$  and  $1 \times 10^3$  cells, respectively). After 3 days, allantoic fluid was recovered from the eggs and virus reconstitution was examined by HA test. To calculate reconstitution efficiency, eggs showing HA activity that were inoculated with  $1 \times 10^5$  cells,  $1 \times 10^4$  cells and  $1 \times 10^3$  cells, were counted as 1, 10, and 100 point(s), respectively.

#### <Results>

Results of Examples 12 and 13 are shown in Figures 40 to 43,

and Table 2. The combination of envelope expressing plasmid and cell overlay increased the reconstitution efficiency of SeV/ $\Delta$ F-GFP. Notable improvement was obtained in d3 to d4 (day 3 to day4) of P0 (before subculture) (Figure 41). In Table 2, eggs were inoculated with cells 3 days after transfection. The highest reconstitution efficiency was obtained in day 3 when treated with 0.3  $\mu$ g/ml psoralen for 20 min. Thus, these conditions were taken as optimal conditions (Table 2).

Table 2: Effect of PLWUV treatment of vaccinia virus on reconstitution of Sendai virus

Effect of PLWUV treatment of vaccinia virus on reconstitution of Sendai virus (eggs were inoculated with cells 3 days after transfection)		The number of HA-positive eggs (b)							
		0 $\mu$ g/ml		0.3 $\mu$ g/ml		1 $\mu$ g/ml		3 $\mu$ g/ml	
		0'	20'	5'	10'	20'	5'	10'	20'
The number of inoculated cells	Score (a)	0	0	3	3	3	3	3	3
$10^5$	1(a1)	0	3	3	3	3	3	3	3
$10^4$	10(a2)	0	3	2	3	3	1	3	1
$10^3$	100(a3)	0	3	0	1	1	0	1	0
Reconstitution Score	$(a1+a2+a3) \times b$	0	333	43	133	133	13	133	13

$$\text{Reconstitution Score} = (a1+a2+a3) \times b$$

GFP-non-comprising Sendai virus vector

<Construction of F-deficient type, LacZ gene-comprising SeV vector cDNA>

To construct cDNA comprising LacZ gene at Not I restriction site existing at the upstream region of NP gene of pSeV18<sup>+</sup>/ΔF described in Example 1 (pSeV (+18:LacZ)/ΔF), PCR was performed to amplify the LacZ gene. PCR was carried out by adjusting LacZ gene to multiples of 6 (Hausmann, S et al., RNA 2, 1033-1045 (1996)) and using primer (5'-GCGCGGCCGCGGTACGGTGGCAACCATGTCGTTTACTTTGACCAA-3'/SEQ ID NO: 17) comprising Not I restriction site for 5' end, and primer (5'-GCGCGGCCGCGATGAACTTTCACCCTAAGTTTTTCTTACTACGGCGTACGCTATTACTTC TGACACCAGACCAACTGGTA-3'/SEQ ID NO: 18) comprising transcription termination signal of SeV (E), intervening sequence (I), transcription initiation signal (S), and Not I restriction site for 3' end, using pCMV-β (Clontech) as template. The reaction conditions were as follows. 50 ng pCMV-β, 200 μM dNTP (Pharmacia Biotech), 100 pM primers, 4 U Vent. polymerase (New England Biolab) were mixed with the accompanying buffer, and 25 reaction temperature cycles of 94°C 30 sec, 50°C 1 min, 72°C 2 min were used. Resultant products were electrophoresed with agarose gel electrophoreses. Then, 3.2 kb fragment was cut out and digested with NotI after purification. pSeV(+18:LacZ)/ΔF was obtained by ligating with NotI digested fragment of pSeV18<sup>+</sup>/ΔF.

<Conventional method>

LLC-MK2 cells were seeded onto 100 mm culture dish at 5x 10<sup>6</sup> cells/dish, and after a 24 hour culture, the cells were washed once with MEM medium without serum. Then, the cells were infected with recombinant vaccinia virus (vTF7-3) (Fuerst, T.R. et al., Proc. Natl. Acad. Sci. USA 83, 8122-8126 (1986)) expressing T7 RNA polymerase at room temperature for 1 hour (moi=2) (moi=2 to 3, preferably moi=2 is used). The virus used herein was pretreated with 3 μg/ml psoralen and long-wave ultraviolet light (365 nm) for 5 min. LacZ comprising, F-deficient type Sendai virus vector cDNA (pSeV(+18:LacZ) ΔF), pGEM/NP, pGEM/P, and pGEM/L (Kato, A. et al., Genes Cells 1, 569-579 (1996)) were suspended in Opti-MEM medium (GIBCO) at a ratio of 12 μg, 4 μg, 2 μg, and 4 μg/dish, respectively, 4 μg/dish envelope plasmid

pGEM/FHN and SuperFect transfection reagent (1  $\mu$ g DNA/5  $\mu$ l, QIAGEN) were added and left to stand at room temperature for 15 min. Then, 3 ml Opti-MEM medium containing 3% FBS was added and the cells were washed twice with MEM medium without serum, and then the DNA-SuperFect mixture was added. After a 3 hr culture, cells were washed twice with MEM medium without serum, and cultured 24 hours in MEM medium containing 40  $\mu$ g/ml cytosine  $\beta$ -D-arabinofuranoside (AraC, Sigma) and 7.5  $\mu$ g/ml trypsin. Culture supernatants were removed and 5 ml of suspension of a 100 mm culture dish of F expressing LLC-MK2/F7 cells in MEM medium without serum (containing 40  $\mu$ g/ml AraC and 7.5  $\mu$ g/ml trypsin) was overlaid onto the cells. After further a 48 hr culture, the cells and supernatants were recovered and designated as P0-d3 samples. The P0-d3 pellets were suspended in Opti-MEM medium ( $2 \times 10^7$  cells/ml) and after 3 times of freeze-thawing, were mixed with lipofection reagent DOSPER (Boehringer Mannheim) ( $10^6$  cells/25  $\mu$ l DOSPER) and left to stand at room temperature for 15 min. Then, F expressing LLC-MK2/F7 cells were transfected with the mixture ( $10^6$  cells/well, 24-well plate) and cultured with MEM medium without serum (containing 40  $\mu$ g/ml AraC and 7.5  $\mu$ g/ml trypsin). The culture supernatants were recovered on day 7, and designated as P1-d7 samples. Further, total volumes of supernatants were infected to F expressing LLC-MK2/F7 cells seeded onto 12-well plates at 37°C for 1 hour. Then, after washing once with MEM medium, the cells were cultured in MEM medium without serum (containing 40  $\mu$ g/ml AraC and 7.5  $\mu$ g/ml trypsin). The culture supernatants were recovered on day 7, and were designated as P2-d7 samples. Further, total volumes of supernatants were infected to F expressing LLC-MK2/F7 cells seeded onto 6-well plates at 37°C for 1 hour. Then, after washing once with MEM medium, the cells were cultured in MEM medium without serum (containing 7.5  $\mu$ g/ml trypsin). The culture supernatants were recovered on day 7, and were designated as P3-d7 samples. Further, total volumes of supernatants were infected to F expressing LLC-MK2/F7 cells seeded onto 10 cm plates at 37°C for 1 hour. Then, after washing once with MEM medium, the cells were cultured in MEM medium without serum (containing 40  $\mu$ g/ml AraC and 7.5  $\mu$ g/ml trypsin). The culture supernatants were recovered on day 7, and were designated as P4-d7 samples.



<Measurement of CIU by counting LacZ-expressing cells (LacZ-CIU)>

LLC-MK2 cells were seeded onto 6-well plate at  $2.5 \times 10^6$  cells/well, and after a 24 hr culture, the cells were washed once with MEM medium without serum and infected with 1/10 fold serial  
 5 dilution series of P3-d7 made using MEM medium at 37°C for 1 hour. Then, the cells were washed once with MEM medium and 1.5 ml MEM medium containing 10% serum was added. After a three day culture at 37°C, cells were stained with  $\beta$ -Gal staining kit (Invitrogen). Result of  
 10 experiment repeated three times is shown in Figure 44. As the result of counting LacZ staining positive cell number,  $1 \times 10^6$  CIU/ml virus was obtained in P3-d7 samples in any case.

[Example 15] Regulation of gene expression levels using polarity effect in Sendai virus

15 <Construction of SeV genomic cDNA>

Additional NotI sites were introduced into Sendai virus (SeV) full length genomic cDNA, namely pSeV(+) (Kato, A. et al., Genes to Cells 1: 569-579, 1996), in between start signal and ATG translation initiation signal of respective genes. Specifically, fragments of  
 20 pSeV(+) digested with SphI/SalI (2645 bp), ClaI (3246 bp), and ClaI/EcoRI (5146 bp) were separated with agarose gel electrophoreses and corresponding bands were cut out and then recovered and purified with QIAEXII Gel Extraction System (QIAGEN) as shown in Figure 45(A). The SphI/SalI digested fragment, ClaI digested fragment, and  
 25 ClaI/EcoRI digested fragment were ligated to LITMUS38 (NEW ENGLAND BIOLABS), pBluescriptII KS+ (STRATAGENE), and pBluescriptII KS+ (STRATAGENE), respectively, for subcloning. Quickchange Site-Directed Mutagenesis kit (STRATAGENE) was used for successive introduction of NotI sites. Primers synthesized and used for each  
 30 introduction were, sense strand:  
 5'-ccaccgaccacacccagcgccgcgacagccacggcttcgg-3' (SEQ ID NO: 19),  
 antisense strand: 5'-ccgaagccgtggctgtcgcgccgctgggtgtggtcggtgg-3'  
 (SEQ ID NO: 20) for NP-P, sense strand:  
 5'-gaaatttcacctaagcggccgcaatggcagatatctatag-3' (SEQ ID NO: 21),  
 35 antisense strand: 5'-ctatagatatctgccattgcgccgcttaggtgaaatttc-3'  
 (SEQ ID NO: 22) for P-M, sense strand:

5'-gggataaagtccttgcgccgcttggttgcaaaactctcccc-3' (SEQ ID NO: 23),  
 antisense strand:  
 5'-ggggagagagttttgcaaccaagcgccgcaagggactttatccc-3' (SEQ ID NO: 24)  
 for M-F, sense strand:  
 5'-ggtcgcgcggtacttttagcgccgcctcaaacaagcacagatcatgg-3' (SEQ ID NO:  
 25), antisense strand:  
 5'-ccatgatctgtgcttggttgaggcgccgctaaagtaccgcgcgacc-3' (SEQ ID NO:  
 26) for F-HN, sense strand:  
 5'-cctgcccattccatgacctagcgccgcttcccattcaccctggg-3' (SEQ ID NO: 27),  
 10 antisense strand:  
 5'-cccagggtgaatgggaagcgccgctaggtcatggatgggcagg-3' (SEQ ID NO: 28)  
 for HN-L.

As templates, SalI/SphI fragment for NP-P, ClaI fragments for  
 P-M and M-F, and ClaI/EcoRI fragments for F-HN and HN-L, which were  
 15 subcloned as described above were used, and introduction was carried  
 out according to the protocol accompanying Quickchange Site-Directed  
 Mutagenesis kit. Resultants were digested again with the same enzyme  
 used for subcloning, recovered, and purified. Then, they were  
 assembled to Sendai virus genomic cDNA. As a result, 5 kinds of  
 20 genomic cDNA of Sendai virus (pSeV(+)NPP, pSeV(+)PM, pSeV(+)MF,  
 pSeV(+)FHN, and pSeV(+)HNL) in which NotI sites are introduced between  
 each gene were constructed as shown in Figure 45(B).

As a reporter gene to test gene expression level, human secreted  
 type alkaline phosphatase (SEAP) was subcloned by PCR. As primers,  
 25 5' primer: 5'-gcggcgcgccatgctgctgctgctgctgctgctgggctg-3' (SEQ ID  
 NO: 29) and 3' primer:  
 5'-gcggcgcgcccttatcatgtctgctcgaagcgccgcccgc-3' (SEQ ID NO: 30)  
 added with AscI restriction sites were synthesized and PCR was  
 performed. pSEAP-Basic (CLONTECH) was used as template and Pfu turbo  
 30 DNA polymerase (STRATAGENE) was used as enzyme. After PCR, resultant  
 products were digested with AscI, then recovered and purified by  
 electrophoreses. As plasmid for subcloning, pBluescriptII KS+  
 incorporated in its NotI site with synthesized double strand DNA  
 [sense strand:  
 35 5'-gcggcgcgcgtttaaacggcgccgcccatttaaatccgtagtaagaaaaacttaggggtgaaagt  
 tcatcgcgccgc-3' (SEQ ID NO: 31), antisense strand:

5'-gcgccgcgatgaactttcaccctaagtttttcttactacggatttaaattggcgcgccggtt  
 taaacgcggccgc-3' (SEQ ID NO: 32)] comprising multicloning site  
 (PmeI-AscI-SwaI) and termination signal-intervening  
 sequence-initiation signal was constructed (Figure 46). To AscI site  
 5 of the plasmid, recovered and purified RCR product was ligated and  
 cloned. The resultant was digested with NotI and the SEAP gene  
 fragment was recovered and purified by electrophoreses to ligate into  
 5 types of Sendai virus genomic cDNA and NotI site of pSeV18+  
 respectively. The resultant virus vectors were designated as  
 10 pSeV(+ )NPP/SEAP, pSeV(+ )PM/SEAP, pSeV(+ )MF/SEAP, pSeV(+ )FHN/SEAP,  
 pSeV(+ )HNL/SEAP, and pSeV18(+ )/SEAP, respectively.

#### <Virus reconstitution>

LLC-MK2 cells were seeded onto 100 mm culture dishes at  $2 \times 10^6$   
 cells/dish, and after 24 hour culture the cells were infected with  
 15 recombinant vaccinia virus (PLWUV-Vact7) (Fuerst, T.R. et al., Proc.  
 Natl. Acad. Sci. USA 83: 8122-8126, 1986, Kato, A. et al., Genes Cells  
 1: 569-579, 1996) expressing T7 polymerase for 1 hour (moi=2) at room  
 temperature for 1 hour, in which the virus was pretreated with psoralen  
 and UV. Each Sendai virus cDNA incorporated with SEAP, pGEM/NP,  
 20 pGEM/P, and pGEM/L were suspended in Opti-MEM medium (GIBCO) at ratio  
 of 12  $\mu$ g, 4  $\mu$ g, 2  $\mu$ g, and 4  $\mu$ g/dish, respectively, 110  $\mu$ l of SuperFect  
 transfection reagent (QIAGEN) was added, and left to stand at room  
 temperature for 15 min and 3 ml Opti-MEM medium containing 3% FBS  
 was added. Then, the cells were washed and DNA-SuperFect mixture was  
 25 added. After a 3 to 5 hour culture, cells were washed twice with MEM  
 medium without serum, and cultured 72 hours in MEM medium containing  
 cytosine  $\beta$ -D-arabinofuranoside (AraC). These cells were recovered  
 and the pellets were suspended with 1 ml PBS, freeze-thawed three  
 times. The 100  $\mu$ l of resultant was inoculated into chicken eggs, which  
 30 was preincubated 10 days, and further incubated 3 days at 35°C, then,  
 allantoic fluid was recovered. The recovered allantoic fluids were  
 diluted to  $10^{-5}$  to  $10^{-7}$  and re-inoculated to chicken eggs to make it  
 vaccinia virus-free, then recovered similarly and stocked in aliquots  
 at -80°C. The virus vectors were designated as SeVNPP/SEAP,  
 35 SeVPM/SEAP, SeVMF/SEAP, SeVFHN/SEAP, SeVHNL/SEAP, and SeV18/SEAP.  
 <Titer measurement by plaque assay>

CV-1 cells were seeded onto 6-well plates at  $5 \times 10^5$  cells/well and cultured for 24 hours. After washing with PBS, cells were incubated 1 hour with recombinant SeV diluted as  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$  and  $10^{-7}$  by BSA/PBS (1% BSA in PBS), washed again with PBS, then overlaid with 3 ml/well of BSA/MEM/agarose (0.2% BSA + 2x MEM, mixed with equivalent volume of 2% agarose) and cultured at  $37^\circ\text{C}$ , 0.5%  $\text{CO}_2$  for 6 days. After the culture, 3 ml of ethanol/acetic acid (ethanol:acetic acid=1:5) was added and left to stand for 3 hours, then removed with agarose. After washing three times with PBS, cells were incubated with rabbit anti-Sendai virus antibody diluted 100-folds at room temperature for 1 hour. Then, after washing three times with PBS, cells were incubated with Alexa Flour™ labeled goat anti rabbit Ig(G+H) (Molecular Probe) diluted 200-folds at room temperature for 1 hour. After washing three times with PBS, fluorescence images were obtained by lumino-image analyzer LAS1000 (Fuji Film) and plaques were measured. Results are shown in Figure 47. In addition, results of titers obtained are shown in Table 3.

Table 3: Results of titers of each recombinant Sendai virus measured from results of plaque assay

Recombinant virus	Titer (pfu/ml)
SeV18/SEAP	$3.9 \times 10^9$
SeVNPP/SEAP	$4.7 \times 10^8$
SeVPM/SEAP	$3.8 \times 10^9$
SeVMF/SEAP	$1.5 \times 10^{10}$
SeVFHN/SEAP	$7.0 \times 10^9$
SeVHNL/SEAP	$7.1 \times 10^9$

#### <Comparison of reporter gene expression>

LLC-MK2 cells were seeded onto a 6-well plate at 1 to  $5 \times 10^5$  cells/well and after a 24 hour culture, each virus vector was infected at moi=2. After 24 hours, 100  $\mu\text{l}$  of culture supernatants was recovered

and SEAP assay was carried out. Assay was accomplished with Reporter Assay Kit -SEAP- (Toyobo) and measured by lumino-image analyzer LAS1000 (Fuji Film). The measured values were indicated as relative values by designating value of SeV18+/SEAP as 100. As a result, SEAP activity was detected regardless of the position SEAP gene was inserted, indicated in Figure 48. SEAP activity was found to decrease towards the downstream of the genome, namely the expression level decreased. In addition, when SEAP gene is inserted in between NP and P genes, an intermediate expression level was detected, in comparison to when SEAP gene is inserted in the upstream of NP gene and when SEAP gene is inserted between P and M genes.

[Example 16] Increase of propagation efficiency of deficient SeV by double deficient  $\Delta$ F-HN overlay method

Since the SeV virus reconstitution method used now utilizes a recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3), a portion of the infected cells is killed by the cytotoxicity of the vaccinia virus. In addition, virus propagation is possible only in a portion of cells and it is preferable if virus propagation could be done efficiently and persistently in a more cells. However, in the case of paramyxovirus, cell fusion occurs when F and HN protein of the same kind virus exists on the cells surface at the same time, causing syncytium formation (Lamb and Kolakofsky, 1996, Fields virology, p1189). Therefore, FHN co-expressing cells were difficult to subculture. Therefore, the inventors thought that recovery efficiency of deficient virus may increase by overlaying helper cells expressing deleted protein (F and HN) to the reconstituted cells. By examining overlaying cells with different times of FHN expression, virus recovery efficiency of FHN co-deficient virus was notably increased.

LLC-MK2 cells ( $1 \times 10^7$  cells/dish) grown to 100% confluency in 10 cm culture dishes was infected with PLWUV-treated vaccinia virus at moi=2 for 1 hour at room temperature. After that, mixing 12  $\mu$ g/10 cm dish, 4  $\mu$ g/10 cm dish, 2  $\mu$ g/10 cm dish, 4  $\mu$ g/10 cm dish, and 4  $\mu$ g/10 cm dish of FHN-deficient cDNA comprising d2EGFP (pSeV18+/ $\Delta$ FHN-d2GFP (Example 8)), pGEM/NP, pGEM/P, pGEM/L, and pGEM/FHN, respectively

(3 ml/10cm dish as final volume), and using gene introduction reagent SuperFect (QIAGEN), LLC-MK2 cells were introduced with genes using a method similar to that as described above for the reconstitution of F-deficient virus. After 3 hours, cells were washed three times with medium without serum, then, the detached cells were recovered by slow-speed centrifugation (1000 rpm/2 min) and suspended in serum free MEM medium containing 40 µg/ml AraC (Sigma) and 7.5 µg/ml trypsin (GIBCO) and added to cells and cultured overnight. FHN co-expressing cells separately prepared, which were 100% confluent 10 cm culture dishes, were induced with adenovirus AxCANCre at MOI=10, and cells at 4 hours, 6 hours, 8 hours, day 2, and day 3 were washed once with 5 ml PBS(-) and detached by cell dissociation solution (Sigma). Cells were collected by slow speed centrifugation (1000 rpm/2 min) and suspended in serum free MEM medium containing 40 µg/ml AraC (Sigma) and 7.5 µg/ml trypsin (GIBCO). This was then added to cells in which FHN co-deficient virus was reconstituted (P0) and cultured overnight. Two days after overlaying the cells, cells were observed using fluorescence microscopy to confirm the spread of virus by GFP expression within the cells. The results are shown in Figure 49. When compared to the conventional case (left panel) without overlaying with cells, notably more GFP-expressing cells were observed when cells were overlaid with cells (right). These cells were recovered, suspended with  $10^7$  cells/ml of Opti-MEM medium (GIBCO) and freeze-thawed for three times to prepare a cell lysate. Then, FHN co-expressing cells 2 days after induction were infected with the lysate at  $10^6$  cells/100 µl/well, and cultured 2 days in serum free MEM medium containing 40 µg/ml AraC (Sigma) and 7.5 µg/ml trypsin (GIBCO) at 37°C in a 5% CO<sub>2</sub> incubator, and the virus titer of culture supernatant of P1 cells were measured by CIU-GFP (Table 4). As a result, no virus amplification effect was detected 4 hours after FHN induction, and notable amplification effects were detected 6 hours or more after induction due to cell overlaying. Especially, viruses released into P1 cell culture supernatant were 10 times more after 6 hours when cell overlaying was done compared to when cell overlaying was not done.

Table 4: Amplification of deficient SeV by double deficient  $\Delta F$ -HN cell overlay method

GFP -CIU		$\times 10^3/\text{ml}$				
		FHNcell+ad/cre				
FHN cell-	4h	6h	8h	2d	3d	
8-10	6-9	80-100	70-100	60-100	20-50	

5 [Example 17] Confirmation of pseudotype Sendai virus's possession of F-deficient genome

Western analysis of proteins of extracts of infected cells was carried out to confirm that the virus propagated by VSV-G gene expression described above is F-deficient type. As a result, 10 proteins derived from Sendai virus were detected, whereas F protein was not detected, confirming that the virus is F-deficient type (Figure 50).

15 [Example 18] Effect of anti-VSV antibody on infectiousness of pseudotype Sendai virus comprising F and HN gene-deficient genome

To find out whether pseudotype Sendai virus comprising F and HN gene-deficient genome, which was obtained by using VSV-G expressing line, comprises VSV-G protein in its capsid, neutralizing activity of whether or not infectiousness is affected was examined 20 using anti-VSV antibody. Virus solution and antibody were mixed and left to stand for 30 min at room temperature. Then, LLCG-L1 cells in which VSV-G expression has not been induced were infected with the mixture and gene-introducing capability on day 4 was analyzed by the existence of GFP-expressing cells. As a result, perfect 25 inhibition of infectiousness was seen by anti-VSV antibody in the pseudotype Sendai virus comprising F and HN gene-deficient genome (VSV-G in the Figure), whereas no inhibition was detected in Sendai virus comprising proper capsid (F, HN in the Figure) (Figure 51). Thus, the virus obtained in the present example was proven to be

pseudotype Sendai virus comprising VSV-G protein as its capsid, and that its infectiousness can be specifically inhibited by the antibody.

[Example 19] Purification of pseudotype Sendai viruses comprising F gene-deficient and F and HN gene-deficient genomes by density gradient ultracentrifugation

Using culture supernatant of virus infected cells, sucrose density gradient centrifugation was carried out, to fractionate and purify pseudotype Sendai virus comprising deficient genomes of F gene and F and HN genes. Virus solution was added onto a sucrose solution with a 20 to 60% gradient, then ultracentrifuged for 15 to 16 hours at 29000 rpm using SW41 rotor (Beckman). After ultracentrifugation, a hole was made at the bottom of the tube, then 300  $\mu$ l fractions were collected using a fraction collector. For each fraction, Western analysis were carried out to test that the virus is a pseudotype Sendai virus comprising a genome deficient in F gene or F and HN genes, and VSV-G protein as capsid. Western analysis was accomplished by the method as described above. As a result, in F-deficient pseudotype Sendai virus, proteins derived from the Sendai virus, HN protein, and VSV-G protein were detected in the same fraction, whereas F protein was not detected, confirming that it is a F-deficient pseudotype Sendai virus. On the other hand, in F and HN-deficient pseudotype Sendai virus, proteins derived from Sendai virus, and VSV-G protein were detected in the same fraction, whereas F and HN protein was not detected, confirming that it is F and HN deficient pseudotype Sendai virus (Figure 52).

[Example 20] Overcoming of haemagglutination by pseudotype Sendai virus comprising F gene-deficient and F and HN gene-deficient genomes

LLC-MK2 cells were infected with either pseudotype Sendai virus comprising F gene-deficient or F and HN gene-deficient genome, or Sendai virus with normal capsid, and on day 3, 1% avian red blood cell suspension was added, and left to stand for 30 min at 4°C. Thereafter, cell surface of infected cells expressing GFP were observed. As a result, for virus with F gene-deficient genome and F-deficient pseudotype Sendai virus (SeV/ $\Delta$ F, and pseudotype



SeV/ $\Delta$ F(VSV-G) by VSV-G), agglutination reaction was observed on the surface of infected cells, as well as for the Sendai virus with the original capsid. On the other hand, no agglutination reaction was observed on the surface of infected cells for pseudotype Sendai virus comprising F and HN gene-deficient genome (SeV/ $\Delta$ F-HN(VSV-G)) (Figure 53).

[Example 21] Infection specificity of VSV-G pseudotype Sendai virus comprising F gene-deficient genome to cultured cells

Infection efficiency of VSV-G pseudotype Sendai virus comprising F gene-deficient genome to cultured cells was measured by the degree of GFP expression in surviving cells 3 days after infection using flow cytometry. LLC-MK2 cells showing almost the same infection efficiency in pseudotype Sendai virus comprising F gene-deficient genome and Sendai virus with original capsid were used as controls for comparison. As a result, no difference in infection efficiency was found in human ovary cancer HRA cells, whereas in Jurkat cells of T cell lineage about 2-fold increase in infection efficiency of VSV-G pseudotype Sendai virus comprising F gene-deficient genome was observed compared to controls (Figure 54).

[Example 22] Construction of F-deficient type Sendai virus vector comprising NGF

<Reconstitution of NGF/SeV/ $\Delta$ F>

Reconstitution of NGF/SeV/ $\Delta$ F was accomplished according to the above described "Envelope plasmid + F expressing cells overlaying method". Measurement of titer was accomplished by a method using anti-SeV polyclonal antibody.

<Confirmation of virus genome of NGF/SeV/ $\Delta$ F (RT-PCR)>

To confirm NGF/SeV/ $\Delta$ F virus genome (Figure 55, upper panel), culture supernatant recovered from LLC-MK2/F7 cells were centrifuged, and RNA was extracted using QIAamp Viral RNA mini kit (QIAGEN) according to the manufacturer's protocol. Using the RNA template, synthesis and PCR of RT-PCR was carried out using SUPERSCRIPT<sup>TM</sup> ONE-STEP<sup>TM</sup> RT-PCR SYSTEM (GIBCO BRL). As control groups, additional type SeV cDNA (pSeV18<sup>+</sup> b(+)) (Hasan, M. K. et al., J. General Virology

78: 2813-2820, 1997) was used. NGF-N and NGF-C were used as PCR primers. For NGF-N, forward:

ACTTGCGGCCGCCAAAGTTCAGTAATGTCCATGTTGTTCTACACTCTG (SEQ ID NO: 33),

and for NGF-C, reverse:

5 ATCCGCGGCCGCGATGAACTTTCACCCTAAGTTTTTCTTACTACGGTCAGCCTCTTCTTGTAGC  
CTTCCTGC (SEQ ID NO: 34) were used. As a result, when NGF-N and NGF-C  
were used as primers, an NGF specific band was detected for NGF/SeV/ $\Delta$ F  
in the RT conditions. No band was detected for the control group  
(Figure 55, bottom panel).

10 [Example 23] NGF protein quantification and measurement of *in vitro*  
activity expressed after infection of F-deficient type SeV comprising  
NGF gene

Infection and NGF protein expression was accomplished using  
15 LLC-MK2/F or LLC-MK2 cells grown until almost confluent on culture  
plates of diameter of 10 cm or 6 cm. NGF/SeV/ $\Delta$ F and NGF/SeV/ $\Delta$ F-GFP  
were infected to LLC-MK2/F cells, and NGF/SeV and GFP/SeV were  
infected to LLC-MK2 cells at m.o.i. 0.01, and cultured 3 days with  
MEM medium without serum, containing 7.5  $\mu$ g/ml trypsin (GIBCO).  
20 After the 3 day culture, in which almost 100% of cells are infected,  
medium was changed to MEM medium without trypsin and serum and further  
cultured for 3 days. Then, each culture supernatant were recovered  
and centrifuged at 48,000x g for 60 min. Then, quantification of NGF  
protein and measurement of *in vitro* activity for the supernatant were  
25 carried out. Although in the present examples, F-deficient type SeV  
(NGF/SeV/ $\Delta$ F, NGF/SeV/ $\Delta$ F-GFP) (see Figure 55) are infected to  
LLC-MK2/F cells, if infected with a high m.o.i. (e.g. 1 or 3), namely,  
infected to cells that are nearly 100% confluent from the beginning,  
experiment giving similar results can be performed using F  
30 non-expressing cells.

For NGF protein quantification, ELISA kit NGF Emax Immuno Assay  
System (Promega) and the accompanying protocol were used. 32.4  $\mu$ g/ml,  
37.4  $\mu$ g/ml, and 10.5  $\mu$ g/ml of NGF protein were detected in NGF/SeV/ $\Delta$ F,  
NGF/SeV/ $\Delta$ F-GFP, and NGF/SeV infected cell culture supernatant,  
35 respectively. In the culture supernatant of NGF/SeV/ $\Delta$ F and  
NGF/SeV/ $\Delta$ F-GFP infected cells, high concentration of NGF protein

exists, similar to culture supernatant of NGF/SeV infected cells, confirming that F-deficient type SeV expresses enough NGF.

The measurement of *in vitro* activity of NGF protein was accomplished by using a dissociated culture of primary chicken dorsal root ganglion (DRG; a sensory neuron of chicken) using survival activity as an index (Nerve Growth Factors (Wiley, New York), pp.95-109 (1989)). Dorsal root ganglion was removed from day 10 chicken embryo, and dispersed after 0.25% trypsin (GIBCO) treatment at 37°C for 20 min. Using high-glucose D-MEM medium containing 100 units/ml penicillin (GIBCO), 100 units/ml streptomycin (GIBCO), 250 ng/ml amphotericin B (GIBCO) 20  $\mu$ M 2-deoxyuridine (Nakarai), 20  $\mu$ M 5-fluorodeoxyuridine (Nakarai), 2 mM L-glutamine (Sigma), and 5% serum, cells were seeded onto 96-well plate at about 5000 cells/well. Polylysine precoated 96-well plates (Iwaki) were further coated with laminin (Sigma) before use. At the start point, control NGF protein or previously prepared culture supernatant after SeV infection was added. After 3 days, cells were observed under a microscope as well as conducting quantification of surviving cells by adding Alamar blue (CosmoBio) and using the reduction activity by mitochondria as an index (measuring 590 nm fluorescence, with 530 nm excitation). Equivalent fluorescence signals were obtained in control (without NGF addition) and where 1/1000 diluted culture supernatant of cells infected with SeV/additional-type-GFP (GFP/SeV) was added, whereas the addition of 1/1000 diluted culture supernatant of cells infected with NGF/SeV/ $\Delta$ F, NGF/SeV/ $\Delta$ F-GFP, and NGF/SeV caused a notable increase in fluorescence intensity, and was judged as comprising a high number of surviving cells and survival activity (Figure 56). The value of effect was comparable to the addition of amount of NGF protein calculated from ELISA. Observation under a microscope proved a similar effect. Namely, by adding culture supernatant of cells infected with NGF/SeV/ $\Delta$ F, NGF/SeV/ $\Delta$ F-GFP, and NGF/SeV, increase in surviving cells and notable neurite elongation was observed (Figure 57). Thus, it was confirmed that NGF expressed after infection of NGF-comprising F-deficient type SeV is active form.

[Example 24] Detailed analysis of F-expressing cells

## 1. moi and induction time course of Adeno-Cre

By using different moi of Adeno-Cre, LLC-MK2/F cells were infected and after induction of F protein, the amount of protein expression and the change in cell shape were analyzed.

5 Expression level was slightly higher in moi=10 compared with moi=1 (Figure 58). When expression amounts were analyzed at time points of 6 h, 12 h, 24 h, and 48 h after induction, high expression level of F protein at 48 h after induction was detected in all cases.

10 In addition, changes in cell shape were monitored in a time course as cells were infected with moi=1, 3, 10, 30, and 100. Although a notable difference was found up to moi=10, cytotoxicity was observed for moi=30 or over (Figure 59).

## 2. Passage number

After induction of F protein to LLC-MK2/F cells using Adeno-Cre, 15 cells were passaged 7 times and expression level of F protein and the morphology of the cells were analyzed using microscopic observation. On the other hand, laser microscopy was used for analysis of intracellular localization of F protein after induction of F protein in cells passaged until the 20<sup>th</sup> generation.

20 For laser microscopic observation, LLC-MK2/F cells induced with F protein expression were put into the chamber glass and after overnight culture, media were removed and washed once with PBS, then fixed with 3.7% Formalin-PBS for 5 min. Then after washing cells once with PBS, cells were treated with 0.1% Triton X100-PBS for 5 min, 25 and treated with anti-F protein monoclonal antibody ( $\gamma$ -236) (1/100 dilution) and FITC labeled goat anti-rabbit IgG antibody (1/200 dilution) in this order, and finally washed with PBS and observed with a laser microscope.

As a result, no difference was found in F protein expression 30 levels in cells passaged up to 7 times (Figure 60). No notable difference was observed in morphological change, infectiousness of SeV, and productivity. On the other hand, when cells passaged up to 20 times were analyzed for intracellular localization of F protein using the immuno-antibody method, no big difference was found up to 35 15 passages, but localization tendency of F protein was observed in cells passaged more than 15 times (Figure 61).

Taken together, cells before 15 passages are considered desirable for the production of F-deficient SeV.

[Example 25] Correlation between GFP-CIU and anti-SeV-CIU

5       Correlation of the results of measuring Cell-Infected Unit (CIU) by two methods was analyzed. LLC-MK2 cells were seeded onto a 12-well plate at  $2 \times 10^5$  cells/dish, and after a 24 hour culture, cells were washed once with MEM medium without serum, and infected with 100  $\mu$ l/well SeV/ $\Delta$ F-GFP. After 15 min, 1 ml/well serum-free MEM  
10       medium was added and further cultured for 24 hours. After the culture, cells were washed three times with PBS(-) and dried up (left to stand for approximately 10 to 15 min at room temperature) and 1 ml/well acetone was added to fix cells and was immediately removed. Cells were dried up again (left to stand for approximately 10 to 15 min  
15       at room temperature). Then, 300  $\mu$ l/well of anti-SeV polyclonal antibody (DN-1) prepared from rabbits and diluted 1/100 with PBS(-) was added to cells and incubated at 37°C for 45 min and washed three times with PBS(-). Then, to the cells, 300  $\mu$ l/well of anti-rabbit IgG(H+D) fluorescence-labeled second antibody (Alex<sup>TM</sup> 568, Molecular  
20       Probes) diluted 1/200 with PBS(-) was added, and incubated at 37°C for 45 min and washed three times with PBS(-). Then, cells with fluorescence were observed under fluorescence microscopy (Emission: 560 nm, Absorption: 645 nm, Filters: Leica).

As a control, cells were infected with 100  $\mu$ l/well of SeV/ $\Delta$ F-GFP  
25       and after 15 min, 1 ml/well of MEM without serum was added. After a further 24 hour culture, GFP-expressing cells were observed under fluorescence microscopy (Emission: 360 nm, Absorption: 470 nm, Filters: Leica) without further manipulations.

A Good correlation was obtained by evaluating the fluorescence  
30       intensity by quantification (Figure 62).

[Example 26] Construction of multicloning site

A multicloning site was added to the SeV vector. The two methods used are listed below.

35       1. Several restriction sites in full-length genomic cDNA of Sendai virus (SeV) and genomic cDNA of pSeV18<sup>+</sup> were disrupted, and another

restriction site comprising the restriction site disrupted was introduced in between start signal and ATG translation initiation signal of each gene.

2. Into already constructed SeV vector cDNA, multicloning site sequence and transcription initiation signal - intervening sequence-termination signal were added and incorporated into NotI site.

In the case of method 1, as an introducing method, EagI-digested fragment (2644 bp), ClaI-digested fragment (3246 bp), ClaI/EcoRI-digested fragment (5146 bp), and EcoRI-digested fragment (5010 bp) of pSeV18<sup>+</sup> were separated by agarose electrophoreses and the corresponding bands were cut out, then it was recovered and purified by QIAEXII Gel Extraction System (QIAGEN). EagI-digested fragment was ligated and subcloned into LITMUS38 (NEW ENGLAND BIOLABS) and ClaI-digested fragment, ClaI/EcoRI-digested fragment, and EcoRI-digested fragment were ligated and subcloned into pBluescriptII KS<sup>+</sup> (STRATAGENE). Quickchange Site-Directed Mutagenesis kit (STRATAGENE) was used for successive disruption and introduction of restriction sites.

For disruption of restriction sites, Sal I: (sense strand)  
 5'-ggagaagtctcaacaccgtccaccaagataatcgatcag-3' (SEQ ID NO: 35),  
 (antisense strand) 5'-ctgatcgattatcttgggtggacggtggttgagacttctcc-3'  
 (SEQ ID NO: 36), Nhe I: (sense strand)  
 5'-gtatatgtgttcagttgagcttgctgtcgggtctaaggc-3' (SEQ ID NO: 37),  
 (antisense strand) 5'-gccttagaccgacagcaagctcaactgaacacatatac-3'  
 (SEQ ID NO: 38), Xho I: (sense strand)  
 5'-caatgaactctctagagaggctggagtcactaaagagttacctgg-3' (SEQ ID NO: 39),  
 (antisense strand)  
 5'-ccaggtaactcttttagtgactccagcctctctagagagttcattg-3' (SEQ ID NO: 40),  
 and for introducing restriction sites, NP-P: (sense strand)  
 5'-gtgaaagttcatccaccgatcggtcactcgaggccacaccaaccccaccg-3' (SEQ ID  
 NO: 41), (antisense strand)  
 5'-cggtgggggttgggtgtggcctcgagtgagccgatcggtggatgaactttcac-3' (SEQ ID  
 NO: 42), P-M: (sense strand)  
 5'-cttagggtgaaagaaatttcagctagcacggcgcaatggcagatatc-3' (SEQ ID NO:  
 43), (antisense strand)  
 5'-gatatctgccattgcgccgtgctagctgaaatttctttcacctaag-3' (SEQ ID NO:

44) , M-F: (sense strand)  
 5'-cttagggataaagtccttgtgcgcgcttggttgcaaaactctcccc-3' (SEQ ID  
 NO:45), (antisense strand)  
 5'-ggggagagagttttgcaaccaagcgcgcacaaagggactttatccctaag-3' (SEQ ID NO:  
 5 46) , F-HN: (sense strand)  
 5'-ggtcgcgcggtacttttagtcgacacctcaaacaagcacagatcatgg-3' (SEQ ID  
 NO:47), (antisense strand)  
 5'-ccatgatctgtgcttgtttgaggtgtcgactaaagtaccgcgcgacc-3' (SEQ ID  
 NO:48) , HN-L: (sense strand)  
 10 5'-cccaggggtgaatgggaagggccggccaggtcatggatgggcaggagtcc-3' (SEQ ID  
 NO: 49) , (antisense strand)  
 5'-ggactcctgcccattccatgacctggccggcccttccattcaccctggg-3' (SEQ ID  
 NO: 50) , were synthesized and used for the reaction. After  
 introduction, each fragment was recovered and purified similarly as  
 15 described above, and cDNA were assembled.

In the case of method 2, (sense strand)  
 5'-ggccgcttaattaacggttttaaacgcgcgccaacagtggttgataagaaaaacttaggggtga  
 aagttcatcac-3' (SEQ ID NO: 51), (antisense strand)  
 5'-ggccgtgatgaactttcaccctaagtttttcttatcaacactggttgcgcgcggttttaaac  
 20 gtttaattaagc-3' (SEQ ID NO: 52), were synthesized, and after  
 phosphorylation, annealed by 85°C 2 min, 65°C 15 min, 37°C 15 min,  
 and room temperature 15 min to incorporate into SeV cDNA.  
 Alternatively, multicloning sites of pUC18 or pBluescriptII, or the  
 like, are subcloned by PCR using primers comprising termination signal  
 25 - intervening sequence - initiation signal and then incorporate the  
 resultant into SeV cDNA. The virus reconstitution by resultant cDNA  
 can be performed as described above.

[Example 27] Effects of culture temperature (32°C) on viral  
 30 reconstitution

To quantify the expression level of the gene comprised in virus,  
 three types of SeV cDNAs as shown in Fig. 63 were used. To construct  
 cDNA comprising a secretory alkaline phosphatase (SEAP) gene, a SEAP  
 fragment (1638 bp) having the termination signal-intervening  
 35 sequence-initiation signal downstream of the SEAP gene was excised  
 using NotI, electrophoresed, purified, recovered, and incorporated

to the NotI site of pSeV18+/ $\Delta$ F-GFP to obtain pSeV18+SEAP/ $\Delta$ F-GFP (Fig. 63).

Viral reconstitution was carried out in a similar manner as described above. In this case, since the virus is deficient in F gene, helper cells to supply F protein are used, and the helper cells are prepared using the Cre/loxP expression inducing system. The system utilizes the plasmid pCALNdLw (Arai, T. et al., J. Virol. 72: 1115-1121 (1998)) designed so as to induce the expression of gene product with Cre DNA recombinase, in which a transformant of the plasmid is infected with a Cre DNA recombinase-expressing recombinant adenovirus (AxCANCre) by the method of Saito, et al. (Saito, I. et al., Nucl. Acid. Res. 23, 3816-3821 (1995); Arai, T. et al., J. Virol. 72, 1115-1121 (1998)) to express inserted genes. In the case of SeV-F protein, the transformant cells containing the F gene are referred to as LLC-MK2/F7, and cells continuously expressing F protein after the induction with AxCANCre are referred to as LLC-MK2/F7/A.

Specifically, the viral reconstitution was carried out as follows. LLC-MK2 cells were plated on 100-mm diameter Petri dishes at  $5 \times 10^6$  cells/dish, cultured for 24 h, and then infected with a recombinant vaccinia virus expressing T7 polymerase, which had been treated with the long-wavelength ultraviolet light (365 nm) for 20 min in the presence of psoralen (PLWUV-VacT7: Fuerst, T. R. et al., Proc. Natl. Acad. Sci. USA 83, 8122-8126 (1986)) at room temperature for 1 h (m.o.i.=2). A plasmid encoding SeV cDNA (Fig. 63), pGEM/NP, pGEM/P, pGEM/L, and pGEM/F-HN (Kato, A. et al., Genes Cells 1, 569-579 (1996)) were suspended in Opti-MEM (Gibco-BRL, Rockville, MD) at weight ratios of 12  $\mu$ g, 4  $\mu$ g, 2  $\mu$ g, 4  $\mu$ g and 4  $\mu$ g/dish, respectively. To the suspension, 1  $\mu$ g DNA/5  $\mu$ l equivalent SuperFect transfection reagent (Qiagen, Bothell, WA) were added and mixed. The mixture was allowed to stand at room temperature for 15 min and finally added to 3 ml of Opti-MEM containing 3% FBS. After the cells were washed with a serum-free MEM, the mixture was added to the cells and the cells were cultured. After cultured for 5 h, the cells were washed twice with a serum-free MEM, and then cultured in MEM containing 40  $\mu$ g/ml of cytosine  $\beta$ -D-arabinofuranoside (AraC: Sigma, St. Louis, MO) and 7.5  $\mu$ g/ml of trypsin (Gibco-BRL, Rockville, MD). After cultured



for 24 h, cells continuously expressing F protein (LLC-MK2/F7/A) were layered at  $8.5 \times 10^6$  cells/dish, and cultured in MEM containing 40  $\mu\text{g/ml}$  of AraC and 7.5  $\mu\text{g/ml}$  of trypsin for further 2 days at 37°C (P0). These cells were recovered, and the pellet was suspended in 2 ml/dish of Opti-MEM. After three repeated cycles of freezing and thawing, the lysate thus obtained was transfected as a whole to LLC-MK2/F7/A cells, and the cells were cultured using a serum-free MEM containing 40  $\mu\text{g/ml}$  of AraC and 7.5  $\mu\text{g/ml}$  of trypsin at 32°C (P1). Five to seven days later, an aliquot of the culture supernatant was sampled and infected to freshly prepared LLC-MK2/F7/A cells, and the cells were cultured using the serum-free MEM containing 40  $\mu\text{g/ml}$  of AraC and 7.5  $\mu\text{g/ml}$  of trypsin at 32°C (P2). Three to five days later, the supernatant was infected again to freshly prepared LLC-MK2/F7/A cells, and the cells were cultured using a serum-free MEM containing only 7.5  $\mu\text{g/ml}$  of trypsin at 32°C for 3 to 5 days (P3). To the culture supernatant thus recovered, BSA was added to make a final concentration of 1%, and the resulting mixture was stored at -80°C. The stored virus solution was thawed and used in subsequent experiments.

Titers of virus solutions prepared by this method were  $3 \times 10^8$  and  $1.8 \times 10^8$  GFP-CIU/ml for SeV18+/ $\Delta$ F-GFP and SeV18+SEAP/ $\Delta$ F-GFP, respectively. In the measurement of these titers, with SeV18+/ $\Delta$ F-GFP, the spread of plaque after its infection to F protein continuously expressing cells (LLC-MK2/F7/A) was examined at 32°C and 37°C. As shown in Fig. 64, representing the micrograph 6 days after the infection, it was demonstrated that the spread of plaques significantly increased with cells cultured at 32°C as compared with those cultured at 37°C. Thus, it has become evident that the reconstitution efficiency is enhanced by performing the SeV reconstitution at 32°C after the stage P1, so that it is highly possible to enable the recovery of virus which has been hitherto difficult to obtain.

Two points are considered as the reason for the enhancement of reconstitution efficiency at 32°C. One point is that cytotoxicity due to AraC supplemented to inhibit the amplification of vaccinia virus is thought to be suppressed in culturing at 32°C as compared

with 37°C. Under the virus reconstituting conditions, when LLC-MK2/F7/A cells were cultured in a serum-free MEM containing 40 µg/ml of AraC and 7.5 µg/ml of trypsin, at 37°C, cell damages were caused already 3 to 4 days later with increased detached cells, while, at 32°C, the culture could be sufficiently continued for 7 to 10 days with the cells kept intact. In the case of reconstitution of SeV with an inferior transcription/replication efficiency or with a poor efficiency for infectious virion formation, the culture duration time is thought to be directly reflected in the achievement of reconstitution. A second point is that the expression of F protein is maintained in LLC-MK2/F7/A cells when the cells are cultured at 32°C. After LLC-MK2/F7/A cells which continuously express F protein were cultured at 37°C to confluency on 6-well culture plates in MEM containing 10% FBS, the medium was replaced with a serum free MEM containing 7.5 µg/ml of trypsin, and the cells were further cultured at 32°C or 37°C. The cells were recovered over time using a cell scraper, and semi-quantitatively analyzed for F protein inside the cells by Western-blotting using an anti-F protein antibody (mouse monoclonal). F protein expression was maintained for 2 days at 37°C, decreasing thereafter, while its expression was maintained at least for 8 days at 32°C (Fig. 65). From these results, the validity of viral reconstitution at 32°C (after P1 stage) has been also confirmed.

The above-described Western-blotting was carried out using the following method. Cells recovered from one well of a 6-well plate were stored at -80°C, then thawed in 100 µl of 1 x diluted sample buffer for SDS-PAGE (Red Loading Buffer Pack; New England Biolabs, Beverly, MA), and heated at 98°C for 10 min. After centrifugation, a 10-µl aliquot of the supernatant was loaded on SDS-PAGE gel (multigel 10/20; Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). After electrophoresis at 15 mA for 2.5 h, proteins were transferred to a PVDF membrane (Immobilon PVDF transfer membrane; Millipore, Bedford, MA) by semi-dry method at 100 mA for 1 h. The transfer membrane was immersed in a blocking solution (Block Ace; Snow Brand Milk Products Co., Ltd., Sapporo, Japan) at 4°C for 1 h or more, then soaked in a primary antibody solution containing 10% Block Ace supplemented with 1/1000 volume of the anti-F protein antibody, and allowed to

stand at 4°C overnight. After washed three times with TBS containing 0.05% Tween 20 (TBST), and further three times with TBS, the membrane was immersed in a secondary antibody solution containing 10% Block Ace and supplemented with 1/5000 volume of the anti-mouse IgG+IgM antibody bound with HRP (Goat F(ab')<sub>2</sub> Anti-Mouse IgG + IgM, HRP; BioSource Int., Camarillo, CA), and stirred at room temperature for 1 h. After the membrane was washed three times with TBST and then three times with TBS, the proteins on the membrane were detected by chemiluminescence method (ECL western blotting detection reagents; Amersham Pharmacia biotech, Uppsala, Sweden).

[Example 28] Quantification of secondarily released virus-like particles from SeV deficient in F gene (HA assay, Western-Blotting)

Together with SeV18+/ $\Delta$ F-GFP, using the autonomously replicating type SeV comprising all the viral proteins and comprising GFP fragment (780 bp) having the termination signal-intervening sequence-initiation signal downstream of the GFP gene at the NotI site (SeV18+GFP: Fig. 63), levels of secondarily released virus-like particles were compared.

To LLC-MK2 cells grown to confluency on 6-well plates,  $3 \times 10^7$  CIU/ml each of virus solutions at 100  $\mu$ l per well were added (m.o.i.=3), and the cells were allowed to be infected for 1 h. After the cells were washed with MEM, a serum-free MEM (1 ml) was added to each well, and the cells were cultured at 32°C, 37°C and 38°C, respectively. Sampling was carried out every day, and immediately after the sampling, 1 ml of the fresh serum-free MEM was added to the remaining cells. Culturing and sampling were performed over time. Observation of GFP expression 3 days after the infection under a fluorescence microscope indicated almost the equal level of infection and similar expression of GFP with both types of viruses and under all the conditions at 32°C, 37°C and 38°C (Fig. 66).

Secondarily released virus-like particles were quantified by the hemagglutination activity (HA activity) assay performed according to the method of Kato et al. (Kato, A., et al., Genes Cell 1, 569-579 (1996)). That is, using plates with round-bottomed 96 wells, the virus solution was serially diluted with PBS to make a serial 2-fold

dilutions in 50  $\mu$ l for each well. To 50  $\mu$ l of the virus solution were added 50  $\mu$ l of a preserved chicken blood (Cosmobio, Tokyo, Japan) diluted to 1% with PBS, and the mixture was allowed to stand at 4°C for 1 h. Then, agglutination of erythrocytes was examined. Among  
5 agglutinated samples, the highest dilution rate to achieve hemagglutination was judged as the HA activity. In addition, one hemagglutination unit (HAU) was calculated as  $1 \times 10^6$  viruses, and the hemagglutination activity was also expressed by the number of virus-like particles (Fig. 67). Although, at lower temperatures,  
10 secondarily released virus-like particles were observed with SeV18+/ $\Delta$ F-GFP, a remarkable decrease in the level of virus-like particle release was detected at 38°C as compared with the autonomously replicating SeV (SeV18+GFP).

To quantify the secondarily released virus-like particles from  
15 another point of view, the quantification thereof by Western-Blotting was performed. In a similar manner as described above, LLC-MK2 cells were infected at m.o.i.=3 with the virus, warmed at 37°C, and the culture supernatant and cells were recovered 2 days after the infection. The culture supernatant was centrifuged at 48,000 g for  
20 45 min to recover the viral proteins. After SDS-PAGE, Western-Blotting was performed to detect proteins with an anti-M protein antibody. This anti-M protein antibody is a newly prepared polyclonal antibody, which has been prepared from the serum of rabbits immunized with a mixture of three synthetic peptides: corresponding  
25 to amino acids 1-13 (MADIYRFPKFSYE+Cys/SEQ ID NO: 53), 23-35 (LRTGPDKKAIPH+Cys/SEQ ID NO: 54), and 336-348 (Cys+NVVAKNIGRIRKL/SEQ ID NO: 55) of SeV-M protein. Western-Blotting was performed according to the method as described in Example 27, in which the primary antibody, anti-M protein antibody, was used at a 1/4000  
30 (1:4000) dilution, and the secondary antibody, anti-rabbit IgG antibody bound with HRP (Anti-rabbit IgG (Goat) H+L conj.; ICN P., Aurola, OH) was used at a 1/5000 (1:5000) dilution. With the autonomously replicating SeV (SeV18+GFP), a large amount of M protein was detected in the culture supernatant. With SeV18+/ $\Delta$ F-GFP, however,  
35 a main portion (70%) of M protein was present in the cells, supporting that, with the F gene-deficient SeV, the release of virus-like

particles is reduced at 37°C as compared with the autonomously replicating SeV (Fig. 68).

[Example 29] Construction of genomic cDNA of M gene deficient SeV having EGFP gene

In this construction, a full-length genomic cDNA of the M-deficient SeV deficient in M gene (pSeV18+/ $\Delta$ M: W000/09700) was used. The construction scheme was shown in Fig. 69. BstEII fragment (2098 bp) comprising the M-deficient site of pSeV18+/ $\Delta$ M was subcloned to the BstEII site of pSE280 (Invitrogen, Groningen, Netherlands), in which EcoRV recognition site had been deleted by the previous digestion with SalI/XhoI followed by ligation (construction of pSE-BstEIIfrg). pEGFP having the GFP gene (TOYOBO, Osaka, Japan) was digested with Acc65I and EcoRI, and the 5'-end of the digest was blunted by filling in using the DNA blunting Kit (Takara, Kyoto, Japan). The blunted fragment was subcloned into pSE-BstEIIfrg that, after digested with EcoRV, had been treated with BAP (TOYOBO, Osaka, Japan). This BstEII fragment containing the EGFP gene was returned to the original pSeV18+/ $\Delta$ M to construct the M gene-deficient SeV genomic cDNA (pSeV18+/ $\Delta$ M-GFP) comprising the EGFP gene at the M-deficient site.

[Example 30] Construction of SeV genomic cDNA deficient in M and F genes

The construction scheme described below is shown in Fig. 70. Using the pBlueNaeIfrg- $\Delta$ FGFP, which had been constructed by subcloning an NaeI fragment (4922 bp) of the F-deficient Sendai virus full-length genome cDNA comprising the EGFP gene at the F gene-deficient site (pSeV18+/ $\Delta$ F-GFP) to the EcoRV site of pBluescript II (Stratagene, La Jolla, CA), the deletion of M gene was carried out. Deletion was designed so as to excise the M gene using the ApaLI site right behind the gene. That is, the ApaLI recognition site was inserted right behind the P gene so that the fragment to be excised becomes 6n (6 nucleotides long). Mutagenesis was performed using the QuickChange<sup>TM</sup> Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the method described in the kit. Sequences of

synthetic oligonucleotides used for the mutagenesis are 5'-agagtcactgaccaactagatcgtgcacgaggcatcctaccatcctca-3'/SEQ ID NO: 56 and 5'-tgaggatggtaggatgcctcgtgcacgatctagttggtcagtgactct-3'/SEQ ID NO: 57. After the mutagenesis, the resulting mutant cDNA was partially digested with ApaLI (at 37°C for 5 min), recovered using the QIAquick PCR Purification Kit (QIAGEN, Bothell, WA), and then ligated as it was. The DNA was recovered again using the QIAquick PCR Purification Kit, digested with BsmI and StuI, and used to transform DH5 $\alpha$  to prepare the M gene-deficient (and F gene-deficient) DNA (pBlueNaeIfrg- $\Delta$ M $\Delta$ FGFP).

pBlueNaeIfrg- $\Delta$ M $\Delta$ FGFP deficient in the M gene (and the F gene) was digested with SalI and ApaLI to recover a fragment (1480 bp) containing the M gene-deficient site. On the other hand, pSeV18+/ $\Delta$ F-GFP was digested with ApaLI/NheI to recover a fragment (6287 bp) containing the HN gene, and these two fragments were subcloned into the SalI/NheI site of Litmus 38 (New England Biolabs, Beverly, MA) (construction of LitmusSalI/NheIfrg- $\Delta$ M $\Delta$ FGFP). A fragment (7767 bp) recovered by digesting LitmusSalI/NheIfrg- $\Delta$ M $\Delta$ FGFP with SalI/NheI and another fragment (8294 bp) obtained by digesting pSeV18+/ $\Delta$ F-GFP with SalI/NheI that did not comprise genes such as the M and HN genes were ligated to construct an M- and F-deficient Sendai virus full-length genome cDNA having the EGFP gene at the deficient site (pSeV18+/ $\Delta$ M $\Delta$ F-GFP). Structures of the M-deficient (and M- and F-deficient) viruses thus constructed were shown in Fig. 71.

#### [Example 31] Preparation of helper cells expressing SeV-F and SeV-M proteins

To prepare helper cells expressing M protein (and F protein), the same Cre/loxP expression induction system as that employed for the preparation of helper cells (LLC-MK2/F7 cells) for F protein was used.

##### <1> Construction of M gene expressing plasmid

To prepare helper cells which induce the simultaneous expression of F and M proteins, the above-described LLC-MK2/F7 cells were used to transfer M gene to these cells by the above-mentioned

system. However, since pCALNdLw/F which was used for the transfer of F gene had the neomycin resistance gene, it was essential to transfer a different drug resistance gene for the use of the cells. Therefore, first, according to the scheme described in Fig. 72, the neomycin resistance gene of the M gene-comprising plasmid (pCALNdLw/M: M gene was inserted at the *Swa*I site of pCALNdLw) was replaced with the hygromycin resistance gene. That is, after pCALNdLw/M was digested with *Hinc*II and *Eco*T22I, a fragment containing M gene (4737 bp) was isolated by electrophoresis on agarose, and the corresponding band was excised and recovered using the QIAEXII Gel Extraction System. At the same time, the pCALNdLw/M was digested with *Xho*I to recover a fragment (5941 bp) containing no neomycin resistance gene, and then further digested with *Hinc*II to recover a fragment (1779 bp). Hygromycin resistance gene was prepared by performing PCR with pcDNA3.1hygro(+) (Invitrogen, Groningen, Netherlands) as the template using a pair of primers: hygro-5' (5'-tctcgagtcgctcggtacgatgaaaaagcctgaactcaccgcgacgtctgtcgag-3'/SEQ ID NO: 58) and hygro-3' (5'-aatgcatgatcagtaaattacaatgaacatcgaaccccagagtcccgccatttcctttgc cctcggaagcagtgctggggcgctc-3')/SEQ ID NO: 59), and recovering the PCR product using the QIAquick PCR Purification Kit, then digesting the product with *Xho*I and *Eco*T22I. pCALNdLw-hygroM was constructed by ligating these three fragments.

<2> Cloning of helper cells which induce the expression of SeV-M (and SeV-F) protein(s)

Transfection was performed using the Superfect Transfection Reagent by the method described in the protocol of the Reagent as follows. LLC-MK2/F7 cells were plated on 60 mm diameter Petri dishes at  $5 \times 10^5$  cells/dish, and cultured in D-MEM containing 10% FBS for 24 h. pCALNdLw-hygroM (5  $\mu$ g) was diluted in D-MEM containing no FBS and antibiotics (150  $\mu$ l in total) and stirred. To the mixture, the Superfect Transfection Reagent (30  $\mu$ l) was added. The mixture was stirred again, and allowed to stand at room temperature for 10 min. Then, to the resulting mixture was added D-MEM containing 10% FBS (1 ml). The transfection mixture thus prepared was stirred, and added to LLC-MK2/F7 cells which had been once washed with PBS. After a 3

h culture in an incubator at 37°C in 5% CO<sub>2</sub> atmosphere, the transfection mixture was removed, and the cells were washed three times with PBS. To the cells, D-MEM containing 10% FBS (5 ml) was added, and then, the cells were cultured for 24 h. After cultured, the cells were detached using trypsin, plated on a 96-well plate at about 5 cells/well dilution, and cultured in D-MEM containing 10% FBS supplemented with 150 µg/ml hygromycin (Gibco-BRL, Rockville, MD) for about 2 weeks. A clone which had propagated from a single cell was cultured to expand to a 6-well plate culture. One hundred and thirty clones in total thus prepared were analyzed in the following.

<3> Analysis of helper cell clones which induce the expression of SeV-M (and SeV-F) protein(s)

One hundred and thirty clones thus obtained were semi-quantitatively analyzed for expression levels of M protein by Western-blotting. Each clone was plated on 6-well plates, and, at its nearly confluent state, infected at m.o.i.=5 with a recombinant adenovirus expressing Cre DNA recombinase (AxCANCre) diluted in MEM containing 5% FBS according to the method of Saito et al. (Saito, I. et al., Nucl. Acid. Res. 23, 3816-3821 (1995); Arai, T. et al., J. Virol. 72, 1115-1121 (1998)). After the culture at 32°C for 2 days, the culture supernatant was removed. The cells were washed once with PBS, and detached using a scraper for recovery. After performing SDS-PAGE by applying 1/10 amount of the cells thus recovered per lane, Western-Blotting was carried out using the anti-M protein antibody according to the method described in Examples 27 and 28. Among 130 clones, those which showed relatively high expression levels of M protein were also analyzed using the anti-F protein antibody (f236: Segawa, H. et al., J. Biochem. 123, 1064-1072 (1998)) by Western-blotting. Both results are described in Fig. 73.

[Example 32] Reconstitution of SeV virus deficient in M gene

Reconstitution of SeV deficient in the M gene (SeV18+/ΔM-GFP) was carried out in conjunction with assessment of clones described in Example 31. That is, it was examined whether the expansion of GFP protein was observed (whether the supply of M protein from cells was achieved) by the addition of P0 lysate of SeV18+/ΔM-GFP to each clone.



Preparation of P0 lysate was carried out according to the method described in Example 27 as follows. LLC-MK2 cells were plated on 100-mm diameter Petri dishes at  $5 \times 10^6$  cells/dish, cultured for 24 h, and then infected at m.o.i.=2 with PLWUV-VacT7 at room temperature for 1 h. Plasmids: pSeV18+/ $\Delta$ M-GFP, pGEM/NP, pGEM/P, pGEM/L, pGEM/F-HN and pGEM/M were suspended in Opti-MEM at weight ratios of 12  $\mu$ g, 4  $\mu$ g, 2  $\mu$ g, 4  $\mu$ g, 4  $\mu$ g and 4  $\mu$ g/dish, respectively. To the suspension, 1  $\mu$ g DNA/5  $\mu$ l equivalent of SuperFect transfection reagent were added and mixed. The mixture was allowed to stand at room temperature for 15 min and finally added to 3 ml of Opti-MEM containing 3% FBS. After the cells were washed with a serum-free MEM, the mixture was added to the cells and the cells were cultured. After a 5 h culture, the cells were washed twice with a serum-free MEM, and cultured in MEM containing 40  $\mu$ g/ml AraC and 7.5  $\mu$ g/ml trypsin. After cultured for 24 h, LLC-MK2/F7/A cells were layered at  $8.5 \times 10^6$  cells/dish, and further cultured in MEM containing 40  $\mu$ g/ml AraC and 7.5  $\mu$ g/ml trypsin at 37°C for 2 days. These cells were recovered, the pellet was suspended in 2 ml/dish Opti-MEM, and P0 lysate was prepared by repeating 3 cycles of freezing and thawing. On the other hand, 10 different clones were plated on 24-well plates, infected, at near confluency, with AxCANCre at m.o.i.=5, and cultured at 32°C for 2 days after the infection. These cells were transfected with P0 lysate of SeV18+/ $\Delta$ M-GFP at 200  $\mu$ l/well each, and cultured using a serum-free MEM containing 40  $\mu$ g/ml AraC and 7.5  $\mu$ g/ml trypsin at 32°C. Spread of GFP protein due to SeV18+/ $\Delta$ M-GFP was observed with #18 and #62 clones (Fig. 74). Especially, the spread was more rapid with #62, which was used in subsequent experiments. Hereafter, as to the cells, those prior to the induction with AxCANCre are referred to as LLC-MK2/F7/M62, and those after the induction which continuously express F and M proteins are referred to as LLC-MK2/F7/M62/A. Preparation of SeV18+/ $\Delta$ M-GFP was continued using LLC-MK2/F7/M62/A cells, and, 6 days after the infection with P2,  $9.5 \times 10^6$ , and, 5 days after the infection with P4,  $3.7 \times 10^7$  GFP-CIU viruses were prepared.

It is thought that, also in this experiment, the recovery of SeV18+/ $\Delta$ M-GFP virus has become possible only because the technical improvement, namely "culturing at 32°C after the P1 stage" as shown

in Example 27 was available. Supply of M protein trans from cells expressing the protein (LLC-MK2/F7/M62/A) may be a cause for the recovery of SeV18+/ΔM-GFP, but the spread was extremely slow so as to be observed finally 7 days after the P1 infection (Fig. 74). That is, these results have supported that, also in the reconstitution experiment of the virus, "culturing at 32°C after the P1 stage" is very effective in reconstituting SeV with an inferior transcription-replication efficiency or with a poor infectious virion forming efficiency.

[Example 33] Productivity of SeV deficient in M gene

Productivity aspect of this M gene-deficient virus was also investigated. LLC-MK2/F7/M62/A cells were plated on 6-well plates and cultured at 37°C. The cells which reached nearly confluence were moved to the environment at 32°C and, one day after, infected at m.o.i.=0.5 with SeV18+/ΔM-GFP. The culture supernatant was recovered over time to be replaced with a fresh medium. Supernatants thus recovered were assayed for CIU and HAU. Four to six days after the infection, the largest amount of viruses was recovered (Fig. 75). Although HAU was maintained even 6 days or more after the infection, cytotoxicity was strongly exhibited at this point, indicating that this hemagglutination was caused by HA protein not originating in virus particles but by the activity of HA protein free or bound to cell debris. That is, it seems advisable to recover the culture supernatant by the fifth day after the infection for collecting the virus.

[Example 34] Structural confirmation of M gene-deficient SeV

The viral gene of SeV18+/ΔM-GFP was confirmed by RT-PCR, and the viral protein by Western-blotting. In RT-PCR, the virus at the P2 stage 6 days after the infection was used. In the RNA recovery from virus solution, QIAamp Viral RNA Mini Kit (QIAGEN, Bothell, WA) was used, and, in the cDNA preparation, Thermoscript RT-PCR System (Gibco-BRL, Rockville, MD) was utilized. Both systems were performed by the methods described in the protocols attached to the kits. As the primer for cDNA preparation, the random hexamer supplied with

the kit was used. To confirm that the product was formed starting from RNA, RT-PCR was performed in the presence or absence of the reverse transcriptase. PCR was performed with the cDNA prepared above as the template using two pairs of primers: one combination of F3593 (5'-ccaatctaccatcagcatcags-3'/SEQ ID NO: 60) on the P gene and R4993 (5'-ttcccttcacatcgactatgacc-3'/SEQ ID NO: 61) on the F gene, and another combination of F3208 (5'-agagaacaagactaaggctacc-3'/SEQ ID NO: 62) similarly on the P gene and R4993. As expected from the gene structure of SeV18+/ $\Delta$ M-GFP, amplifications of 1073 bp and 1458 bp DNAs were observed from the former and latter combinations, respectively (Fig. 76). In the case of the reverse transcriptase being omitted (RT-), no amplification of the gene occurred, and in the case of M gene being inserted in stead of GFP gene (pSeV18+GFP), 1400 bp and 1785 bp DNAs were amplified, respectively, clearly different in size from the results described above, supporting that this virus is of an M gene deficient structure.

Confirmation in terms of protein was also performed by Western-blotting. LLC-MK2 cells were infected at m.o.i.=3 with SeV18+/ $\Delta$ M-GFP, SeV18+/ $\Delta$ F-GFP and SeV18+GFP, respectively, and the culture supernatants and cells were recovered 3 days after the infection. The culture supernatant was centrifuged at 48,000 g for 45 min to recover viral proteins. After SDS-PAGE, Western-blotting was performed to detect proteins using the anti-M protein antibody, anti-F protein antibody, and DN-1 antibody (rabbit polyclonal) which mainly detects NP protein according to the method described in Examples 27 and 28. Since, in cells infected with SeV18+/ $\Delta$ M-GFP, M protein was not detected while F or NP protein was observed, it was also confirmed in terms of protein that this virus had the structure of SeV18+/ $\Delta$ M-GFP (Fig. 77). In this case, F protein was not observed in cells infected with SeV18+/ $\Delta$ F-GFP, while all the virus proteins examined were detected in cells infected with SeV18+GFP. In addition, as to the virus proteins in the culture supernatant, very little amount of NP protein was observed in the case of infection with SeV18+/ $\Delta$ M-GFP, indicating that there was no or very little secondarily released virus-like particle.

[Example 35] Quantitative analysis concerning the presence or absence of secondarily released virus-like particles of M gene-deficient SeV

As described in Example 34, LLK-MK2 cells were infected at m.o.i.=3 with SeV18+/ $\Delta$ M-GFP, and the culture supernatant was recovered 3 days after the infection, filtered through an 0.45  $\mu$ m pore diameter filter, and centrifuged at 48,000 g for 45 min to recover virus proteins, which were subjected to Western-blotting to semi-quantitatively detect virus proteins in the culture supernatant. As the control, samples, which had been similarly prepared from cells infected with SeV18+/ $\Delta$ F-GFP, were used. Serial dilutions of respective samples were prepared, and subjected to Western-blotting to detect proteins using the DN-1 antibody (primarily recognizing NP protein). The viral protein level in the culture supernatant of cells infected with SeV18+/ $\Delta$ M-GFP was estimated to be about 1/100 that of cells infected with SeV18+/ $\Delta$ F-GFP (Fig. 78). Furthermore, HA activities of the samples were 64 HAU for SeV18+/ $\Delta$ F-GFP versus <2 HAU for SeV18+/ $\Delta$ M-GFP.

Time courses were also examined for the same experiments. That is, LLC-MK2 cells were infected at m.o.i.=3 with SeV18+/ $\Delta$ M-GFP, and the culture supernatant was recovered over time (every day) to measure HA activity (Fig. 79). Four days or more after the infection, HA activity was detected, though little. However, the measurement of LDH activity, an indicator of cytotoxicity, for the sample revealed a clear cytotoxicity caused 4 days or more after the infection in the SeV18+/ $\Delta$ M-GFP-infected cells (Fig. 80), indicating a high possibility that the elevation of HA activity was not due to virus-like particles, but due to the activity by HA protein bound to or free from cell debris. Furthermore, the culture supernatant obtained 5 days after the infection was examined using cationic liposomes, Dosper Liposomal Transfection Reagent (Roche, Basel, Switzerland). That is, the culture supernatant (100  $\mu$ l) was mixed with Dosper (12.5  $\mu$ l), allowed to stand at room temperature for 10 min, and transfected to LLC-MK2 cells cultured to confluency on 6-well plates. Inspection under a fluorescence microscope 2 days after the transfection revealed that many GFP-positive cells were observed in the supernatant of cells infected with SeV18+/ $\Delta$ F-GFP which contained secondarily released

virus-like particles, while very few or almost no GFP-positive cell was observed in the supernatant of cells infected with SeV18+/ $\Delta$ M-GFP (Fig. 81). From the above results, it was able to conclude that the secondary release of virus-like particles could be almost completely suppressed by the deficiency of M protein.

[Example 36] Reconstitution of SeV deficient in both F and M genes

Reconstitution of SeV deficient in both F and M genes (SeV18+/ $\Delta$ M $\Delta$ F-GFP) was performed by the same method for the reconstitution of SeV18+/ $\Delta$ M-GFP as described in Example 32. That is, LLC-MK2 cells were plated on 100-mm diameter Petri dishes at  $5 \times 10^6$  cells/dish, cultured for 24 h, and then infected at m.o.i.=2 with PLWUV-Vact7 at room temperature for 1 h. Plasmids: pSeV18+/ $\Delta$ M $\Delta$ F-GFP, pGEM/NP, pGEM/P, pGEM/L, pGEM/F-HN and pGEM/M were suspended in Opti-MEM at weight ratios of 12  $\mu$ g, 4  $\mu$ g, 2  $\mu$ g, 4  $\mu$ g, 4  $\mu$ g and 4  $\mu$ g/dish, respectively. To the suspension, 1  $\mu$ g DNA/5  $\mu$ l equivalent of SuperFect transfection reagent were added and mixed. The mixture was allowed to stand at room temperature for 15 min and finally added to 3 ml of Opti-MEM containing 3% FBS. After the cells were washed with a serum-free MEM, the mixture was added to the cells and the cells were cultured. After a 5 h culture, the cells were washed twice with a serum-free MEM, and cultured in MEM containing 40  $\mu$ g/ml AraC and 7.5  $\mu$ g/ml trypsin. After cultured for 24 h, LLC-MK2/F7/M62/A cells were layered at  $8.5 \times 10^6$  cells/dish, and further cultured in MEM containing 40  $\mu$ g/ml AraC and 7.5  $\mu$ g/ml trypsin at 37°C for 2 days. These cells were recovered, the pellet was suspended in 2 ml/dish of Opti-MEM, and P0 lysate was prepared by repeating 3 cycles of freezing and thawing. On the other hand, LLC-MK2/F7/M62/A cells were plated on 24-well plates, moved, at near confluency, to the environment at 32°C, and cultured for 1 day. These cells thus prepared were transfected with P0 lysate of SeV18+/ $\Delta$ M $\Delta$ F-GFP at 200  $\mu$ l/well each, and cultured using a serum-free MEM containing 40  $\mu$ g/ml AraC and 7.5  $\mu$ g/ml trypsin at 32°C. With P0, well spread GFP positive cells were observed. With P1, a spread of GFP positive cells was also observed, though very weak (Fig. 82). In the case where LLC-MK2/F7/M62/A cells were infected with SeV18+/ $\Delta$ F-GFP or

SeV18+/ $\Delta$ M-GFP, a smooth spread of GFP positive cells was observed with both viruses (Fig. 83). Cells expressing both F and M (LLC-MK2/F7/M62/A cells) were infected with SeV18+/ $\Delta$ F-GFP or SeV18+/ $\Delta$ M-GFP at m.o.i.=0.5. Three and six days later, sampling was carried out, and the sample was mixed with 1/6.5 volume of 7.5 % BSA (final concentration = 1%) and stored. Productivity of vectors was investigated by measuring the titers. As a result, SeV18+/ $\Delta$ F-GFP was recovered as virus solution of  $10^8$  or more GFP-CIU/ml and SeV18+/ $\Delta$ M-GFP was recovered as virus solution of  $10^7$  or more GFP-CIU/ml (Table 5). That is, these results indicated that M and F proteins can be supplied successfully from the cells.

Table 5

	3 days after infection	6 days after infection	
SeV18+/ $\Delta$ F-GFP	$1.0 \times 10^8$	$1.7 \times 10^8$	
SeV18+/ $\Delta$ M-GFP	$1.0 \times 10^7$	$3.6 \times 10^7$	GFP-CIU

[Example 37] Helper cells improved to express SeV-F and M proteins  
In the case of using M and F-expressing LLC-MK2/F7/M62/A cells as helper cells, virus particles of both M- and F-deficient (M and F-deficient) SeV (SeV18+/ $\Delta$ M $\Delta$ F-GFP) could not be recovered. However, it was possible to reconstitute and produce both F-deficient SeV (SeV18+/ $\Delta$ F-GFP) and M-deficient SeV (SeV18+/ $\Delta$ M-GFP), suggesting that the Cre/loxP expression inducing system in the helper cells is basically capable of trans supply of both M and F proteins. To effectively use the Cre/loxP expression inducing system and reconstitute both M- and F-deficient SeV, it was necessary to further increase amounts of M and F proteins expressed using this system.

#### <1> Constitution of M and F expression plasmid

To enable helper cells to simultaneously induce the expression of M and F proteins, the above-described LLC-MK2/F7/M62 cells that had been already prepared was improved by introducing M and F genes into these cells so as to function under the Cre/loxP expression

inducing system. Since pCALNdLw/F used for the F gene transduction carried the *neo<sup>r</sup>* gene and pCALNdLw/hygrom used for the M gene transduction carried the hygromycin resistance gene, a different drug resistance gene should be used for the additional genes to be introduced into the above cells. According to the scheme described in Fig. 84, the *neo<sup>r</sup>* gene of the F gene-carrying plasmid (pCALNdLw/F: pCALNdLw containing F gene at *Swa*I site) was replaced with the Zeocin resistance gene. Namely, after pCALNdLw/F was digested with *Spe*I and *Eco*T22I, a fragment (5477 bp) containing the F gene was separated by agarose electrophoresis, and the corresponding band excised from the gel was recovered using a QIAEXII Gel Extraction System. Separately, another pCALNdLw/F was cleaved with *Xho*I to recover a fragment (6663 bp) containing no *neo<sup>r</sup>* gene, which was further digested with *Spe*I to recover a 1761 bp fragment. The Zeocin resistance gene was prepared by performing PCR using pcDNA3.1Zeo(+) (Invitrogen, Groningen, Netherlands) as a template and a pair of primers: zeo-5' (5'- TCTCGAGTCGCTCGGTACGatggccaagttgaccagtgccggttcggtgctcac-3'/ SEQ ID NO: 65) and zeo-3' (5'- AATGCATGATCAGTAAATTACAATGAACATCGAACCCAGAGTCCCGCtcagtcctgctcctcg gccacgaagtgcacgcagttg-3'/ SEQ ID NO: 66). The PCR product was recovered using a QIAquick PCR Purification Kit followed by digestion with *Xho*I and *Eco*T22I. pCALNdLw-zeoF was constituted by ligating these three fragments. Then, pCALNdLw-zeoM was constructed by recombining the drug resistance gene-containing fragment of pCALNdLw/hygrom with the *Xho*I fragment containing the Zeocin resistance gene.

## <2> Cloning of helper cells

Transfection was carried out using a LipofectAMINE PLUS reagent (Invitrogen, Groningen, Netherlands) as described below according to the method described in the attached protocol. LLC-MK2/F7/M62 cells were placed in 60-mm Petri dishes at  $5 \times 10^5$  cells/dish, and cultured in D-MEM containing 10% FBS for 24 h. pCALNdLw-zeoF and pCALNdLw-zeoM (1  $\mu$ g each, 2  $\mu$ g in total) were diluted in D-MEM containing no FBS and antibiotics (total volume: 242  $\mu$ l), and, after stirring, LipofectAMINE PLUS reagent (8  $\mu$ l) was added thereto. The

resulting mixture was stirred and allowed to stand at room temperature for 15 min. Then, LipofectAMINE reagent (12  $\mu$ l) previously diluted in D-MEM containing no FBS and antibiotics (250  $\mu$ l in total) was added, and the mixture was allowed to stand at room temperature for 15 min.

5 Furthermore, D-MEM (2 ml) containing no FBS and antibiotics was added, and, after stirring, the transfection mixture thus prepared was added to LLC-MK2/F7/M62 cells which had been washed once in PBS. After a 3-h culturing at 37°C in a 5% CO<sub>2</sub> incubator, D-MEM containing 20% FBS (2.5 ml) was added to the culture without removing the transfection

10 mixture, and the cells were further incubated for 24 h. After the culture, cells were detached using trypsin, plated on 96-well plates at about 5 cells/well or 25 cells/well dilution, and cultured in D-MEM containing 10% FBS supplemented with 500  $\mu$ g/ml Zeocin (Gibco-BRL, Rockville, MD) for about 2 weeks. A clone which had propagated from

15 a single cell was cultured to expand to a 6-well culture plate. Ninety-eight clones in total thus prepared were analyzed in the following.

Ninety-eight clones thus obtained were semi-quantitatively analyzed for expression levels of M and F proteins by Western blotting.

20 Each clone was plated on 12-well plates, and, at its nearly confluent state, infected at m.o.i. = 5 with a recombinant adenovirus expressing Cre DNA recombinase (AxCANCre) diluted in MEM containing 5% FBS according to the method of Saito et al. (Saito, I. et al., Nucl. Acid. Res. 23, 3816-3821 (1995); Arai, T. et al., J. Virol. 72, 1115-1121

25 (1998)). After culturing at 32°C for 2 days, the culture supernatant was removed. The cells were washed once with PBS, detached using a scraper, and recovered. After performing SDS-PAGE by applying 1/5 amount of the cells thus recovered per lane, Western-Blotting was carried out using the anti-M antibody and anti-F antibody (f236:

30 Segawa, H. et al., J. Biochem. 123, 1064-1072 (1998)). Among the 98 clones analyzed, results of 9 clones are shown in Fig. 85.

[Example 38] Reconstitution of SeV deficient in both M and F genes

Reconstitution of SeV deficient in both M and F genes

35 (SeV18+/ $\Delta$ M $\Delta$ F-GFP) was carried out and the assessment of clones described in Example 37 was confirmed. That is, it was assessed



whether the reconstitution of SeV18+/ $\Delta$ M $\Delta$ F-GFP could be achieved using P0 lysate (lysate of transfected cells). P0 lysate was prepared as follows. LLC-MK2 cells were plated on 100-mm diameter Petri dishes at  $5 \times 10^6$  cells/dish, cultured for 24 h, and then infected at m.o.i. = 2 with PLWUV-Vact7 at room temperature for 1 h. Plasmids pSeV18+/ $\Delta$ M $\Delta$ F-GFP, pGEM/NP, pGEM/P, pGEM/L, pGEM/F-HN and pGEM/M were suspended in Opti-MEM at weight ratios of 12  $\mu$ g, 4  $\mu$ g, 2  $\mu$ g, 4  $\mu$ g, 4  $\mu$ g and 4  $\mu$ g/dish, respectively. SuperFect transfection reagent (1  $\mu$ g DNA/5  $\mu$ l equivalent) was added to the suspension and mixed. The mixture was allowed to stand at room temperature for 15 min and added to 3 ml of Opti-MEM containing 3% FBS. After the cells were washed with a serum-free MEM, the mixture was added to the cells and cultured. After a 5-h culturing, the cells were washed twice with a serum-free MEM and cultured in MEM containing 40  $\mu$ g/ml AraC and 7.5  $\mu$ g/ml trypsin. After culturing for 24 h, LLC-MK2/F7/A cells were layered at  $8.5 \times 10^6$  cells/dish, and these cells were further cultured in MEM containing 40  $\mu$ g/ml AraC and 7.5  $\mu$ g/ml trypsin at 37°C for 2 days. These cells were recovered, the pellet was suspended in 2 ml/dish Opti-MEM, and P0 lysate was prepared by repeating 3 cycles of freezing and thawing. Separately, newly cloned cells were plated on 24-well plates, infected, at near confluency, with AxCANCre at m.o.i. = 5, and cultured at 32°C for 2 days after the infection. These cells were transfected with P0 lysate of SeV18+/ $\Delta$ M $\Delta$ F-GFP at 200  $\mu$ l/well each, and cultured using a serum-free MEM containing 40  $\mu$ g/ml AraC and 7.5  $\mu$ g/ml trypsin at 32°C. Spread of GFP protein was observed in 20 clones examined, indicating the successful recovery of M and F-deficient SeV. Results of virus reconstitution in several clones among those examined are shown in Fig. 86. Especially, in the clone #33 (LLC-MK2/F7/M62/#33), infectious virions having the titer of  $10^8$  GFP-CIU/mL or more were recovered at its p3 stage (passaged three times), indicating that this clone is highly promising as a virus producing cell. These results reveal that the introduction of both M and F genes into LLC-MK2/F7/M62 cells successfully prepared cells from which M and F-deficient SeV can be recovered at a high frequency. It is considered that the original LLC-MK2/F7/M62 cells expressed M and F proteins at a sufficient level, and that the recovery of M and F-deficient

SeV has become possible by introducing both M and F genes into the cells, thereby slightly raising the M and F protein expression levels.

[Example 39] Virus productivity from M and F-deficient SeV

5       The virus productivity of this M and F-deficient SeV was also investigated. LLC-MK2/F7/M62/#33 cells were placed in 6-well plates and cultured at 37°C. The cells at near confluency were infected at a MOI of 5 with AxCANCre (LLC-MK2/F7/M62/#33/A), and cultured at 32°C for 2 days after the infection. Then, the cells were infected at a  
10       MOI of 0.5 with SeV18+/ΔMΔF-GFP, and the culture supernatant was recovered at intervals and replaced with a fresh medium. Supernatants thus recovered were examined for their CIU and HAU. On and after the second day of infection, viruses having the titer of 10<sup>8</sup> CIU/ml or more were continuously recovered (Fig. 87).  
15       Furthermore, the time-course changes in CIU and HAU were parallel to each other, and most of virus particles produced had infectivity, indicating the efficient virus production.

20       [Example 40] Confirmation of the structure of M gene- and F gene-deficient SeV

      The viral gene of SeV18+/ΔMΔF-GFP was confirmed by RT-PCR, and the viral protein by Western-blotting. In RT-PCR, the virus at the P2 stage 5 days after the infection (P2d5) was used. RNA was recovered from virus solution using QIAamp Viral RNA Mini Kit (QIAGEN, Bothell,  
25       WA), and cDNA preparation and RT-PCR, was performed using SuperScript One-Step RT-PCR System (Gibco-BRL, Rockville, MD), according to the methods described in the attached protocols. PCR was performed using, as the primer for cDNA preparation and RT-PCR, two pairs of primers: one combination of F3208 (5'-agagaacaagactaaggctacc-3'/SEQ  
30       ID NO: 62) on the P gene and GFP-RV (5'-cagatgaacttcagggtcagcttg-3'/SEQ ID NO: 67) on the GFP gene, and another combination of said F3208 and R6823 (5'-tggtggaatgagagaatcagc-3'/SEQ ID NO: 68) on the HN gene. As  
35       expected from the gene structure of SeV18+/ΔMΔF-GFP, amplifications of 644 bp and 1495 bp DNAs were observed from the former and latter combinations (Fig. 88). Furthermore, from SeV18+/ΔM-GFP and

SeV18+/ $\Delta$ F-GFP, genes in size expected from their respective structures were amplified, and their sizes were clearly different from those obtained from SeV18+/ $\Delta$ M $\Delta$ F-GFP, supporting that SeV18+/ $\Delta$ M $\Delta$ F-GFP lacks both of M and F genes.

5 This was also confirmed by the protein level by Western-blotting. LLC-MK2 cells were infected at m.o.i.=3 with SeV18+/ $\Delta$ M $\Delta$ F-GFP, SeV18+/ $\Delta$ M-GFP, SeV18+/ $\Delta$ F-GFP and SeV18+GFP, and the cells were recovered 2 days after the infection. After SDS-PAGE, Western-blotting was performed according to the method described in  
10 Examples 27 and 28 to detect proteins using the anti-M antibody, anti-F antibody, and DN-1 antibody (rabbit polyclonal) that mainly detects NP protein. In cells infected with SeV18+/ $\Delta$ M $\Delta$ F-GFP, both M and F proteins were not detected while NP protein was observed. Thus, the protein level examination also confirmed the structure of  
15 SeV18+/ $\Delta$ M $\Delta$ F-GFP (Fig. 89). In this experiment, F protein was not observed in cells infected with SeV18+/ $\Delta$ F-GFP, and M protein was not observed in cells infected with SeV18+/ $\Delta$ M-GFP, while all the viral proteins examined were detected in cells infected with SeV18+GFP.

20 [Example 41] Quantitative analysis of the presence or absence of secondarily released particles of SeV deficient in M- and F- genes

Time courses were also examined for the same experiments. Specifically, LLC-MK2 cells were infected at m.o.i.=3 with SeV18+/ $\Delta$ M $\Delta$ F-GFP, and the culture supernatant was recovered over time  
25 (every day) to measure HA activity (Fig. 90). Four days or more after the infection, very little HA activity was detected. This elevation of HA activity was thought to be probably not due to virus-like particles, but due to HA protein bound to or free from cell debris, similar to the case of SeV18+/ $\Delta$ M-GFP. Furthermore, the culture supernatant obtained 5 days after the infection was examined using  
30 cationic liposomes, Dosper Liposomal Transfection Reagent (Roche, Basel, Switzerland). Specifically, the culture supernatant (100  $\mu$ l) was mixed with Dosper (12.5  $\mu$ l), allowed to stand at room temperature for 10 min. The resulting mixture was used to transfect LLC-MK2 cells  
35 cultured to confluency on 6-well plates. Inspection under a fluorescence microscope 2 days after the transfection revealed that

many GFP-positive cells were observed for the supernatant of cells infected with SeV18+/ $\Delta$ F-GFP which contained secondarily released particles, while very few or almost no GFP-positive cell was observed for the supernatant of cells infected with SeV18+/ $\Delta$ M $\Delta$ F-GFP (Fig. 91).  
 5 This result indicates that the cells transfected with SeV18+/ $\Delta$ M $\Delta$ F-GFP contains almost no secondarily released virus particles.

[Example 42] Viral infectivity of M and F-deficient SeV and M-deficient SeV (*in vitro*)

10 Efficiency of introduction of gene transfer vector into non-dividing cells and intracellular expression efficiency are important and essential for the assessment of the capability of the vector.

Primary cultures of rat cerebral cortex nerve cells were  
 15 prepared by the following method. Pregnant SD rat was anesthetized by ether and decapitated on the 17<sup>th</sup> day after conception. After disinfecting the abdomen with isodine and 80% ethanol, the uterus was transferred into a 10-cm Petri dish, and the fetus (embryo) was taken out. Next, the scalp and cranial bone of fetus were cut with  
 20 a pair of INOX5 tweezers, the brain was picked up and collected in a 35-mm diameter Petri dish. Portions of the cerebellum and brain stem were removed with a pair of oculist scissors, the cerebrum was divided into hemispheres, the remaining brain stem was removed, olfactory bulb was taken out with a pair of tweezers, and then the  
 25 meninx was removed also using a pair of tweezers. Finally, after the removal of diencephalon and hippocampus using a pair of oculist scissors, the cerebral cortex was collected in a Petri dish, cut into small pieces with a surgical knife, and collected into a 15-mm centrifuge tube. The cortex was treated with 0.3 mg/ml papain at 37°C  
 30 for 10 min, treated in a serum-containing medium (5 ml), and washed. The cells were then dispersed. The cells were strained through a 70- $\mu$ m strainer, collected by centrifugation, dispersed by gentle pipetting, and then counted. The cells were placed in poly-L-lysine (PLL)-coated 24-well culture plates at  $2 \times 10^5$  or  $4 \times 10^5$  cells/well,  
 35 and, 2 days after seeding, infected at MOI of 3 with M and F-deficient SeV (SeV18+/ $\Delta$ M $\Delta$ F-GFP) and M-deficient SeV (SeV18+/ $\Delta$ M-GFP).

Thirty-six hours after the infection, the cells were immuno-stained with the nerve cell-specific marker MAP2, and infected cells were identified by merging with GFP-expressing cells (SeV-infected cells).

Immunostaining with MAP2 was carried out as follows. After infected cells were washed with PBS, the cells were fixed with 4% paraformaldehyde at room temperature for 10 min, washed in PBS, and then blocked using PBS containing 2% normal goat serum at room temperature for 60 min. Next, the cells were reacted with a 1/200-fold diluted anti-MAP2 antibody (Sigma, St. Louis, MO) at 37°C for 30 min, washed with PBS, and then reacted with a 1/200-fold diluted secondary antibody (goat anti mouse IgG Alexa568: Molecular Probes Inc., Eugene, OR) at 37°C for 30 min. After the cells were washed with PBS, fluorescence intensity of the cells was observed under a fluorescence microscope (DM IRB-SLR: Leica, Wetzlar, Germany).

In both M and F-deficient SeV (SeV18+/ $\Delta$ MAF-GFP) and M-deficient SeV (SeV18+/ $\Delta$ M-GFP), almost all MAP2-positive cells were GFP-positive (Fig. 92). That is, nearly all the prepared nerve cells were efficiently infected with SeV, confirming that both M and F-deficient SeV and M-deficient SeV are highly effectively introduced into non-dividing cells and expressed the transgenes

[Example 43] Viral infectivity of M and F-deficient SeV and M-deficient SeV (*in vivo*)

M and F-deficient SeV (SeV18+/ $\Delta$ MAF-GFP) and M-deficient SeV (SeV18+/ $\Delta$ M-GFP), whose *in vivo* infectivity was evaluated as described above, (5  $\mu$ l) ( $1 \times 10^9$  p.f.u./ml) were intraventricularly administered into the left ventricle of a gerbil using the stereo method. Two days after the administration, the brain was surgically excised to prepare frozen slices. These slices were observed under a fluorescence microscope to examine the presence or absence of infection based on the fluorescence intensity of GFP. By the administration of both M and F-deficient SeV (SeV18+/ $\Delta$ MAF-GFP) and M-deficient SeV (SeV18+/ $\Delta$ M-GFP), many GFP-positive cells were observed among cells in both left and right ventricles, such as ependymal cells (Fig. 93). This result confirmed that both M and F-deficient and M-deficient SeVs enables efficient gene transfer and expression of the transgene

*in vivo*.

[Example 44] Cytotoxicity of M and F-deficient SeV and M-deficient SeV

5. Viral cytotoxicity was assessed using CV-1 and HeLa cells in which SeV infection-dependent cytotoxicity could be observed. As a control, cytotoxicity of SeV having replicability (wild type: SeV18+GFP) and F-deficient SeV (SeV18+/ $\Delta$ F-GFP) was also measured. Experimental procedures are described in detail below. CV-1 cells or HeLa cells were placed in 96-well plates at  $2.5 \times 10^4$  cells/well (100  $\mu$ l/well) and cultured. MEM containing 10% FBS was used for culturing both cells. After culturing for 24 h, the cells were infected by adding at 5  $\mu$ l/well a solution of SeV18+GFP, SeV18+/ $\Delta$ F-GFP, SeV18+/ $\Delta$ M-GFP or SeV18+/ $\Delta$ M $\Delta$ F-GFP diluted with MEM containing 1% BSA, and, 6 h later, the culture medium containing the virus solution was removed, and replaced with MEM medium containing no FBS. Three days after the infection, the culture supernatant was sampled, and the cytotoxicity was quantified using a Cytotoxicity Detection Kit (Roche, Basel, Switzerland) according to the method described in the instruction attached to the kit. Comparing to SeV having the replicability, deficiency in M or F gene attenuated cytotoxicity (as in SeV18+/ $\Delta$ F-GFP and SeV18+/ $\Delta$ M-GFP), and deficiency in both genes (as in SeV18+/ $\Delta$ M $\Delta$ F-GFP) additively attenuated cytotoxicity (Fig. 94).

25. As described above, "M and F-deficient SeV vector" that has been successfully reconstituted for the first time in the present invention, has the infectivity against a variety of cells including non-dividing cells, contains almost no secondarily released virus particles, and, furthermore, has attenuated cytotoxicity. Thus, the vector of this invention can be a gene transfer vector with a wide range of applicability.

#### Industrial Applicability

35. The present invention provides envelope gene-deficient paramyxovirus vectors. The present invention establishes a practical, novel, envelope gene-deficient vector system based on a

negative-strand RNA virus for the first time. The achievement in recovering infectious deficient virus particles from cDNA of F gene-deficient, M gene-deficient, and FHN gene-deficient genome and so on using helper cells, paved the way for the research and development of novel vectors for gene therapy, taking advantage of the excellent characteristics of the Sendai virus. The deficient type Sendai virus vector in the present invention is capable of introducing a gene into various cell types with an extremely high efficiency and expressing the exogenous gene at a phenomenally high level. Furthermore, the vector is expressed in infected cells persistently, and is a highly safe vector that completely lacks the capability to cause virus propagation, since it does not release secondary infectious virus particles. The vector provided by the present invention is expected to be variously applied as a vector for *in vivo* and *ex vivo* gene therapy.

## CLAIMS

1. A paramyxovirus vector comprising a complex comprising (a) a paramyxovirus-derived negative-strand single-stranded RNA modified not to express at least one envelope protein of paramyxoviruses, and (b) proteins that bind to said negative-strand single-stranded RNA.
2. The vector according to claim 1, wherein the negative-strand single-stranded RNA expresses NP protein, P protein, and L protein, and is modified not to express F, HN, or M protein, or any combination thereof.
3. The vector according to claim 1, comprising at least one of the envelope proteins whose expression was suppressed in the modified negative-strand single-stranded RNA.
4. A vector according to claim 1, comprising VSV-G protein.
5. A vector according to claim 1, wherein the negative-strand single-stranded RNA is derived from Sendai virus.
6. A vector according to claim 1, wherein the negative-strand single-stranded RNA further encodes an exogenous gene.
7. A DNA encoding negative-strand single-stranded RNA comprised in a vector according to any one of claims 1 to 6, or the complementary strand thereof.
8. A method for producing a vector according to claim 1, comprising the following steps of:
  - (a) expressing vector DNA encoding a paramyxovirus-derived negative-strand single-stranded RNA modified not to express at least one envelope protein of paramyxoviruses, or the complementary strand, by introducing into cells expressing at least one envelope protein,
  - (b) culturing said cells, and,
  - (c) recovering the virus particles from the culture supernatant.
9. A method for producing a vector according to claim 1, comprising the steps of,
  - (a) introducing, a complex comprising a paramyxovirus-derived negative-strand single-stranded RNA modified not to express at least one envelope protein of paramyxoviruses, and a protein binding to said negative-strand single-stranded RNA, into cells expressing at least one envelope protein,



(b) culturing said cells, and,

(c) recovering virus particles from the culture supernatant.

10. The method according to claim 8 or 9, wherein the cell culture in (b) is a co-culture with cells expressing at least one envelope protein.

11. The method according to claim 8 or 9, wherein cells expressing at least one envelope protein are overlaid to said cells in cell culture in (b).

12. The method of claim 8 or 9, wherein the cell culture is carried out at 35°C or less.

13. A method according to claim 8 or 9, wherein at least one envelope protein expressed by the cells is identical to at least one envelope protein whose expression is suppressed in the negative-strand single-stranded RNA described above.

14. A method according to claim 8 or 9, wherein at least one envelope protein expressed by the cells is VSV-G protein.

**ABSTRACT**

F gene-deficient virus virions are successfully recovered by using an F gene-deficient Sendai virus genomic cDNA. Further, F gene-deficient infectious viral particles are successfully constructed by using F-expressing cells as helper cells. Also, F gene and HN gene-deficient virus virions are successfully recovered by using a virus genomic cDNA deficient in both F gene and HN gene. Further, F gene and HN gene-deficient infectious viral particles are successfully produced by using F- and HN-expressing cells as helper cells. A virus deficient in F gene and HN gene and having F protein is constructed by using F-expressing cells as helper cells. In addition, M gene-deficient infectious virus particles were produced using helper cells expressing M protein. From cells infected with M gene-deficient viruses, release of virus-like particles was inhibited. Further, a VSV-G pseudo type virus is successfully constructed by using VSV-G-expressing cells. Techniques for constructing these deficient viruses contribute to the development of vectors of Paramyxoviridae usable in gene therapy.

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☒ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**